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**Enrichment of minority DNA in admixes of DNA samples:
potential use in non-invasive prenatal diagnosis (NIPD), of
Down syndrome**

By

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Enrichment of minority DNA in admixes of DNA samples: potential use in non-invasive prenatal diagnosis (NIPD), of Down syndrome

Abstract

Background

Non-invasive prenatal diagnosis (NIPD) is a promising approach that is currently being developed. The principle is that fetal material can be detected in maternal plasma and potentially enable women to pursue reliable and timely prenatal diagnosis, whilst eliminating the risk of miscarriage associated with chorionic villus sampling or amniocentesis. However NIPD research has been restricted up until now for the diagnosis of Down syndrome due to the low concentration of free fetal DNA (ffDNA) in maternal plasma. Various methods have been developed in an attempt to increase the concentration of ffDNA.

Methods

This study uses COLD-PCR (co-amplification at lower denaturation temperature PCR) to analyse potential enrichment of ffDNA over maternal DNA through optimization of the critical denaturation temperature (Td), using Real Time-PCR in an attempt to selectively enrich smaller fetal DNA fragments. Fake fetal DNA was created in two different spike experiments to imitate the natural environment of viable ffDNA. One spike experiment used 5% of fake fetal DNA in a 95% maternal background to represent levels of ffDNA during early pregnancy. The other spike experiment utilized 10% of fake fetal DNA in 90% maternal background to denote late pregnancy. Before running COLD-PCR, various adjustments took place to find the critical Td at which one could run the spike experiment by COLD-PCR. Products of spike experiments were analysed on a genetic analyser for fragment analysis. Melt curve analysis was also performed for the spike experiment to identify the specificity of each sample at each denaturation temperatures.

Results

A critical Td (80°C) was identified for the D21S1890 region of chromosome 21 by COLD-PCR. This temperature does allow enrichment of fetal DNA, as fake maternal DNA was undetermined by RT-PCR compared to fake fetal DNA. The spike experiments clearly showed amplification of fake fetal DNA from the mixture of fake fetal and fake maternal DNA at the critical Td of 80°C. Running same samples of spike experiment on the genetic analyser identified peaks from all samples at a Td of 95°C, while at a critical Td of 80°C the result showed decreased numbers of maternal peaks, regardless of stutter peaks formation. Melt curve analysis results clearly identified heteroduplex formation in the samples at the critical Td of 80°C.

Conclusion

The results represent a good indication for using COLD-PCR in enriching ffDNA for detection by RT-PCR. However, as each individual has only two alleles, the observed results of multiple peaks for fragment analysis were not expected. Further research needs to focus on both eliminating heteroduplex formation and stutter peaks. COLD-PCR has the potential to open a new gateway in NIPD for aneuploidy detection. This method could be particularly useful in the detection of genetic abnormalities in the fetus, in particular Down syndrome and other aneuploidies.

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List of abbreviations

Abbreviation	Description
ABI	Applied Biosystems
bp	Base pairs
BSA	Bovine serum albumin
CAH	Congenital adrenal hyperplasia
cffDNA	Cell free fetal DNA
COLD-PCR	Co amplification at lower denaturation temperature polymerase chain reaction
Ct	Cycle threshold
CVS	Chorionic Villus Sampling
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
DS	Down syndrome
EDTA	Ethylenediaminetetra acetic acid
<i>E. coli</i>	<i>Escherichia coli</i>
gDNA	Genomic DNA
NHSBT	National Health Service Blood and Transplant
NIPD	Non-invasive prenatal diagnosis
NRBC	Nucleated red blood cell
NT	Nuchal translucency
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
RT-PCR	Real Time Polymerase Chain Reaction
SNPs	Single nucleotide polymorphisms
STRs	Short tandem repeats
TAE	Tris-acetate-EDTA
Tc	Critical temperature
Td	Denaturation temperature
Tm	Melting temperature
UVP	Ultra-Violet Products
WGA	Whole genome amplification

Dedication

This thesis is dedicated to my parents

Dr. Zhian & Dr. Dara Miran

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Author's Declaration

At no time during the registration for the degree of Research Master has the author been registered for any other University award without prior agreement of the Graduate Committee.

A programme of advanced study was undertaken, which included a course in cellular basis of immunity and postgraduate courses on research skills and methods.

Conferences attended at Plymouth University included a Postgraduate Society conference and one on Translational Biomedicine; and CNAPS VII conference (Circulatory Nucleic Acids in Plasma and Serum) was attended in Madrid.

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1 Chapter One

1.1 Introduction

Prenatal diagnosis is a part of an established obstetrical practice; however conventional methods of prenatal diagnosis to obtain fetal tissues for genetic analysis, including amniocentesis and chorionic villus sampling (CVS), are invasive and constitute a finite risk to the unborn fetus (Evans and Wapner, 2005). Currently used screening tests for aneuploidy are based on the assessment of fetal sonographic markers and/or the elevation of biochemical markers in the maternal circulation during the first and second trimesters. By far the most common aneuploidy compatible with life is Down syndrome (DS, trisomy 21), in which the fetus inherits an extra copy of chromosome 21. The current screening protocol for trisomy 21 is composed of several stages, including blood tests for numerous maternal protein markers and a nuchal translucency (NT) ultrasound scan of the fetus. Prenatal assessment with biochemical markers at 11-13 weeks of gestation, may detect 90-94% of pregnancies affected by trisomy 21 at a false positive rate of 5% (Kagan et al., 2008b). The current gold standard for diagnosis of trisomy 21 is provided by invasive sampling of fetal genetic material through CVS or amniocentesis, followed by conventional cytogenetic or DNA analysis. However, both procedures are associated with an increased risk of fetal loss of about 1%, hence they are only recommended for pregnancies with a high risk of fetal trisomy 21 (Cochrane et al., 2003). Since 1997, when cell free fetal DNA (cffDNA) in maternal circulation was discovered (Lo et al., 1997), the research

interest has focused on the development of reliable techniques for non-invasive prenatal diagnosis (NIPD) that would allow direct analysis of fetal genetic material based on the discovery of cffDNA in the maternal circulation. To date cffDNA has been used to identify fetuses where paternally inherited alleles require detection, for example, male fetuses or *RHD* positive fetuses in D negative mothers. For conditions where maternal alleles require analysis, this has been more challenging due to the high maternal DNA background compared to fetal DNA in maternal plasma. This is regarded as the major limitation to achieve routine NIPD for the common chromosomal abnormalities such as aneuploidy. For the purposes of prenatal diagnosis, detection of aneuploidy requires not just detection but also accurate quantification of the DNA derived from specific chromosome.

Results from techniques such as digital PCR, in which enriched single template DNA molecules are isolated by dilution and then amplified (Lo et al., 2007a), and direct high-throughput shotgun sequencing, in which millions of sequence tags are selected across the genome (Fan et al., 2008) suggest extremely high accuracy. The use of massively parallel sequencing technologies has shown early promise as a method of detection for trisomy 21 (Chiu and Lo, 2011; Faas et al., 2012; Dan et al., 2012; Lo, 2012). Efforts to enrich for cffDNA from chromosome of interest prior to massively parallel sequencing have been described but to date these approaches have not been used for detection of any genetic conditions. Sparks et al., (2012) developed a method called digital analysis of selected region (DANSRTM), which selectively evaluates specific genomic fragments from cfDNA. By enabling selective analysis of cfDNA, DANSR would provide more efficient use of sequencing to evaluate fetal aneuploidy.

It is considered that minor enrichment of cfDNA from a mixture of maternal free DNA and cfDNA may lead to simple detection of ratios of fetal chromosomes by digital counting technology (Lo et al., 2007a), and also it would be cheaper and quicker, whereas next generation sequencing approach is highly expensive and requires substantial technical and bioinformatics input and analysis. In an effort to reduce the cost of sequencing, Liao et al., (2011) have demonstrated that a solution based target capture system is able to focus the sequencing power to selected genomic regions. Such system is able to increase the sequencing coverage of the targeted region by over 200-fold. Therefore, target enrichment provides a logical approach for more efficient and cost-effective massively parallel sequencing because it increases the proportion of informative data from the target region.

Therefore there is a need to find an approach for fetal DNA enrichment. A modified form of PCR known as co-amplification at lower denaturation temperature (COLD-PCR) was reported by (Li et al., 2008). COLD-PCR could selectively amplify minority alleles from background of wild type allele. It exploits subtle difference in the melting temperature (T_m) of variant and mismatched sequences compared to wild-type ones and uses a critical denaturation temperature (T_c) lower than the melting temperature to selectively amplify minority mutated alleles in a wild-type allele background. Li et al (2008) suggested that COLD-PCR could also be used for the detection of fetal alleles in maternal blood. COLD-PCR was originally applied to the detection of tumour-associated mutations and the first application to NIPD has been recently described by (Galbiati et al., 2011).

Our current research is investigating methods to enrich the percentage of cfDNA present in maternal plasma so that conditions, such as trisomy 21, could be assessed using sensitive techniques such as digital PCR. Relying on the size difference between fetal fragment (300 bp) and maternal fragments (>500 bp), our experiments are aiming to exploit this difference to enrich cfDNA from maternal plasma samples. By lowering the denaturation temperature in a PCR reaction, we aim to specifically melt the cfDNA and not the maternal DNA, hence amplify certain regions of the genome from the specific cfDNA pool, without contamination from maternal DNA. Thus by selecting diagnostic regions of certain genes such as short tandem repeats (STR), fetal DNA can be amplified selectively and be used diagnostically using conventional molecular testing such as real time PCR (RT-PCR). A common molecular technique for the amplification and detection of trisomy has focused on the use of the polymorphic STRs. The majority of DNA markers with STR consist of length polymorphisms of di-, tri- or tetranucleotide repeats. It was proposed that STR polymorphism could be applied to detect aneuploidies (Liou et al., 2004). D21S1890 is one of various polymorphic DNA markers located on chromosome 21 that contains a STR. Cytosine, adenine (CA) repeat was chosen as the most useful STR to focus on in this study due to the high variable number of repeats which are non-functional and therefore selectively neutral (Mansfield, 1993). However studies have identified that PCR amplification of dinucleotide repeat polymorphism are difficult to interpret due to the stuttering bands (Kalaitzidaki et al., 1992).

1.2 Research aims and objectives

This project aimed to develop methods for enrichment of free fetal DNA in maternal plasma for NIPD purposes. The objectives were as follows:

- ❖ To develop a method for the enrichment of fetal DNA in maternal plasma through COLD-PCR. Reducing the temperature of denaturation (Td) allows fetal DNA targets to be melted but not the maternal free DNA, due to the size difference. Thus optimization of Td by COLD-PCR is to determine the precise temperature at which fetal fragment DNA being selectively amplified but not maternal DNA fragment.
- ❖ To run different genomic DNA samples on end-point PCR for fragment analysis to determine maternal DNA in preparation for spiking experiments using capillary electrophoresis.
- ❖ For the project, we could not use clinically-derived cffDNA and thus we used a spike experiment where we created a mixture of 5-10% of artificial cffDNA in a pool of 95-90% of maternal circulating DNA. This was used to imitate the cffDNA in a background of maternal DNA during early and late pregnancy. Therefore spiking experiment would be performed using fake fetal DNA fragment and fake maternal DNA, at the same time using same samples on the end-point PCR with FAM labelled primers using capillary electrophoresis for fragment analysis. This would be a step toward developing a reliable technique to identify cffDNA in maternal plasma. It is hoped that we would have a new rapid, cheap and safer choice for the millions of pregnant women worldwide that are considering prenatal diagnosis each year.

1.3 Literature review:

Prenatal diagnosis of fetal chromosomal abnormalities represents a major step in pregnancy management, allowing stepwise planning and co-ordinated care during delivery and the neonatal period. It is mainly performed for detection of fetal genetic and chromosomal abnormalities.

Trisomy 21 is one of the main indications for prenatal diagnosis; it is also the most common cause of mental retardation and has an incidence of about 1:700 to 1:800 live births (Driscoll & Gross, 2009). The condition is associated with intellectual impairments, severe learning difficulties and excess mortality caused by long term health problems such as heart disease, leukaemia and thyroid dysfunction. It results from the presence of three copies of human chromosome 21. The presence of the extra copy of chromosome 21 causes the physical and mental abnormalities characteristic of trisomy 21. Physically, babies with trisomy 21 tend to have certain features: poor Moro reflex, excess skin fold on the back of neck (in neonate), single crease in the palm of hand (simian crease). Other phenotypical features are flat nasal bridge, small mouth, protruded tongue and low-set ear. The condition is either due to nondisjunction (94% of cases), translocation (3.5% of cases) or mosaicism (2.5% of cases) (Chiu and Lo, 2011, Roizen and Patterson, 2003). For the common non-disjunction type of trisomy 21, the incidence varies markedly with maternal age at the time of conception. At a maternal age of 20 years the risk is approximately 1:1450, while at 30 years of age the risk is 1:940 and it can reach as high as 1:85 by the age of 40 (Morris et al., 2003).

1.3.1 Prenatal screening tests

Screening for trisomy 21 should be offered to all pregnant women; it is suggested to be performed by the end of the first trimester (13 weeks) (NICE, 2010). Women are offered a screening test to determine whether an invasive diagnostic test (amniocentesis or CVS) is required for the definitive diagnosis. The screening test does not determine the presence of a chromosome abnormality per se; it only identifies high risk pregnancies (Graaf et al., 2002).

First trimester screening test includes maternal serum biochemical screening and ultrasound measurement of the nuchal translucency.

1.3.2 Maternal serum biochemical screening

Maternal serum biochemical screening includes biochemical measurement of free beta-human chorionic gonadotrophin (β -hCG) and pregnancy associated plasma protein A (PAPP-A) (NICE, 2010). They maximize the detection of trisomy 21 during the first trimester; the detection rate is around 90-95% of pregnancies affected by trisomy 21, at a false positive rate of 5% (Macri and Spencer., 1996). The performance is better at 9 to 10 weeks than at 13 weeks because the difference in PAPP-A between trisomic and euploid pregnancies is greater in earlier gestation (Kagan et al., 2008a, Wright et al., 2010). However these screening tests detect phenotypic features instead of genetic pathology and they must be conducted within a strict gestational age window (11-14 weeks) (Malone et al., 2005). Risk can be assessed by several factors, including elevated or depressed serum levels of these biomarkers, as well as maternal age (Bhide and Thilaganathan, 2004).

1.3.3 Ultrasonography (NT)

A nuchal translucency scan is a screening test which measures the fluid filled area at the back of neck between 11 weeks 0 day and 13 weeks 6 days of pregnancy, the measurements include only the skin fold from the outer edge of the occipital bone to the outer skin edge of the baby. Antenatal screening advice that if the measurement of fluid is greater than 3.5 mm, women should be referred for chromosomal testing. However false positive rates are high (5%) plus most fetal defects are caused by abnormal genetic make-up (chromosomal number, morphology and gene defect) and as such many of them cannot be detected via ultrasound scan (Huang et al., 2011).

There are no significant association between fetal NT and maternal serum free β -hCG or PAPP-A in either trisomy 21 or euploid pregnancies, and therefore the ultrasonographic and biochemical markers can be combined to provide more effective screening than either method individually (Spencer et al., 1999). In addition to NT, other highly sensitive and specific first-trimester sonographic markers of trisomy 21 are absence of nasal bone, increased impedance to flow in the ductus venous and tricuspid regurgitation (Nicolaidis, 2011).

Assessment of each of these ultrasound markers can be incorporated into first trimester combined screening by fetal NT and serum free β -hCG and PAPP-A, resulting in improvement of the performance of screening with an increase in detection rate to 96% and a decrease in false positive rate to 2.5 % (Kagan et al., 2009). On the other hand, it has been estimated that the second trimester scan can improve the detection rate of trisomy 21 achieved by first trimester combined screening (Krantz et al., 2007).

1.3.4 Invasive prenatal tests

An abnormal screening result means high risk (cut-off of 1 in 150) and suggests the need for more invasive procedures such as CVS and amniocentesis (Avent et al., 2009, Bhide and Thilaganathan, 2004). Additionally, women with a history of previous premature babies or babies with birth defects especially genetic problems or a family history of a chromosomal disorder, are usually offered invasive diagnostic tests (Graaf et al., 2002).

1.3.5 Chorionic villus sampling

CVS is used most frequently to identify chromosomal problems, such as trisomy 21. The main advantage of CVS over amniocentesis is that the procedure can be performed much earlier in pregnancy i.e. before 14th gestational week, rather than 16 to 18 weeks. CVS involves removing a tiny piece of tissue from placenta. Under ultrasound guidance, the tissue is obtained either with a needle through abdomen or a catheter inserted through the cervix. The tissue is then cultured and a karyotype analysis is performed (Huang et al., 2011).

1.3.6 Amniocentesis

Amniocentesis is typically performed after the 15th week of gestation. Also under ultrasound guidance, a needle is inserted through the abdomen to remove a small amount of amniotic fluid. Karyotype analysis will then be completed.

Invasive procedures such as amniocentesis from which the full karyotype is usually determined using cultured cells, the two week period needed for cultivation and subsequent analysis has proven to be associated with considerable parental anxiety and medical problem in those situations requiring therapeutic intervention. In order to overcome these problems, a more rapid

method for prenatal diagnosis of fetal chromosomal aneuploidy needed to be developed. A multi-colour Fluorescence in situ hybridization technique (FISH) commercially introduced for uncultured cells, although this method was reliable and has proven in large scales studies to be very accurate, it is time and labour-intensive procedure. Moreover this method requires intact cells, as it can only be used on fresh or specially stored samples (Zimmermann et al., 2006, Thilaganathan et al., 2000).

Amniocentesis and CVS are both associated with a risk of miscarriage about 1-2% as mentioned before and of course added discomfort and stress to mother (Mujezinovic and Alfirevic, 2008). Other risks that may associate with amniocentesis are introduction of pathogens into the amniotic sac from needle, and also possibly preterm labour and delivery.

1.3.7 Non-invasive prenatal diagnosis

Performing prenatal diagnosis non-invasively would be the most promising approach for prenatal diagnosis. In order to perform NIPD, a source of fetal genetic material that could be sampled without harm to fetus would be needed. Many researchers in non-invasive prenatal testing have focused on the isolation and analysis of both fetal nucleated cells and cfDNA that have entered into the maternal circulation.

1.3.8 Free fetal cells in maternal circulation

In 1893 the presence of fetal cells in maternal circulation was documented for the first time when multinucleated syncytial trophoblasts were found in the lung tissue of pregnant women who died from pre-eclampsia (Lapaire et al., 2007). Intact fetal cells, compared to cfDNA have the advantage of retaining complete fetal genetic information in the nucleus and cytoplasm that can provide information in complex genetic diagnosis (Bhide and Thilaganathan, 2004, Graaf et al., 2002). The presence of fetal cells in maternal blood in normal pregnancies has been estimated at one to two cells per ml (Reading *et al.*, 1995). Similar results have been reported by other investigators (Cheung et al., 1996, Bianchi et al., 1997). A considerable challenge for the researchers in this field is to enrich or isolate these rare fetal cells, either for cytogenetic analysis by FISH or for analysis of fetal cell DNA by other molecular techniques (Bianchi and Hanson, 2006).

Various fetal cell types found in maternal blood have been explored as potential candidates for enrichment and subsequent analysis, including fetal trophoblasts, leukocytes and nucleated erythrocytes. Trophoblast enrichment is hindered not only by limited availability of antibodies specific to placental

antigens, but also by multinucleated morphology (Schueler et al., 2001). Leukocytes may persist from previous pregnancies, and isolation strategies are also limited by the lack of availability of unique cell markers (Ciaranfi et al., 1977). Nucleated red blood cells (NRBC) have the advantage of a relatively short half-life of 25-35 days; thus, cells of fetal origin are unlikely to persist from previous pregnancies. NRBC are also relatively abundant in first trimester blood, with unique cell morphology and complete chromosomal complement (Bianchi et al., 1990). However, a growing concern has been expressed that only a small proportion of the recovered NRBC are actually of fetal origin. Moreover, many of these cells are undergoing normal physiological apoptosis, and therefore not surprisingly giving rise to unstable or fragmented DNA that is not suitable for NIPD (Hristoskova et al., 2001).

Bianchi et al. (2002) and Babochkina et al. (2005) demonstrated that the detection of fetal trisomy from these fetal nucleated erythrocyte cells is difficult; possibly due to the fact that the chromosomes in these cells disintegrate some time before the nucleus is eliminated from the cell, making FISH analysis of samples from maternal circulation unreliable. Therefore because these cells are extremely rare and/or may persist for years after prior pregnancies, cffDNA, which is more plentiful in the maternal circulation and unique to the current pregnancy, may have greater potential for use in prenatal diagnosis.

1.3.9 cffDNA in maternal plasma

Between the 1970s and 1990s, a group of researchers were studying the possibility of finding cell free DNA in the plasma of human subjects. One particularly interesting line of research was the detection of tumour derived DNA in the plasma of women suffering from a variety of cancers. Hence, tumour associated oncogene mutations have been detected in the plasma DNA of women with cancer (Chen et al., 1996, Nawroz et al., 1996). Inspired by such work, Lo et al. hypothesized in 1997 that a fetus might also release its DNA in cell-free form into the plasma of its mother. This discovery has opened up new possibilities for NIPD.

The presence cffDNA in maternal plasma was first discovered through the detection of Y-chromosome specific sequence in the plasma of women who were carrying a male fetus (Lo et al., 1997). The presence of cffDNA in maternal plasma is regarded as a major source of fetal genetic material. It constitutes about 3-6% of total free DNA, around 3% in early pregnancy and rises to 6% in late pregnancy (Lo et al., 1998) but the level is high in cases of multiple pregnancies, placental complications, as well as pre-eclampsia (Lo and Chiu, 2008, Hahn and Chitty, 2008). Pregnancies with trisomy 21 exhibit 1.7 fold higher levels of maternal serum cffDNA than controls and also the levels increase in trisomy 13 pregnancies but not in trisomy 18 pregnancies (Lo et al., 1999). cffDNA has been detected in maternal plasma as early as the fourth week of gestation (Alberry and Soothill, 2008). In contrast to nucleated fetal cells, cffDNA has been showed to be cleared from maternal circulation extremely rapidly and it has a short half-life of only 16-28 minute (Rijnders et al., 2004). In one clinical condition, which is maternal liver disease, significant levels of fetal DNA remain in circulation even after delivery (Avent et al., 2009). The

majority of cffDNA exists in small fragments that are less than 300 bp, whereas maternal DNA is more than 500 bp (Chan et al., 2004).

This discovery opened up a new field for NIPD, many studies stated that they did not observe presence of cffDNA in the blood from non-pregnant women whom had experienced previous pregnancies (Rijnders et al., 2004). Those results were also confirmed by a study of Bianchi et al. (2002) when they observed that there is no *SRY* gene in the sera of women carrying female fetus with a history of previous male infant. However another study reported persistence of fetal DNA several years after delivery (Invernizzi et al., 2002). The main hypothesis to explain discordant results of this study is that there may have been a potential contaminant or error in the preparation of samples and measurements plus the use of improper statistical analysis (Mazouni et al., 2007).

1.3.10 Sources of cffDNA

Contrary to popular belief that the placenta forms an impermeable barrier between mother and child, there is bidirectional traffic between the fetus and mother during pregnancy. Multiple studies have shown that both intact fetal cells and cffDNA cross the placenta and circulate in the maternal blood stream. Potential sources of cffDNA include the fetal nucleated red blood cells which undergo apoptosis in the maternal circulation but the most likely source of origin is the placenta (Alberry et al., 2007).

Both serum and plasma can be used as sources for cffDNA but the fractional concentration of cffDNA in maternal serum has been found to be lower than that in maternal plasma. This is possibly due to liberation of DNA from maternal blood cells during the clotting process (Lo, 2000).

1.3.11 Limitations (problems) associated with detecting cffDNA in the maternal circulation

- ❖ cffDNA is usually detected using a Y-chromosome gene (*SRY*), which is not present in the maternal genome, and such assays can be used only in approximately 50% of pregnancies in which the fetus is male (Alberry and Soothill, 2008).
- ❖ The concentration of cffDNA is relatively low.
- ❖ Total amount of cffDNA varies between individuals (Wright and Burton, 2009).
- ❖ The fetus inherits half of its genome from the mother (Wright and Burton, 2009).
- ❖ The size fraction of fetal DNA (300 bp) extracted from maternal blood is smaller than maternal DNA (>500 bp)

1.4 Applications of cffDNA

1.4.1 Fetal rhesus RhD genotyping

Presence of cffDNA has enabled NIPD of paternally inherited alleles that are absent in maternal genome. It is of clinical relevance to determine the fetal *RHD* status prenatally because *RHD* positive fetuses from *RHD* negative mothers are at a high risk of haemolytic disease of the fetus and newborn (HDFN). The screening tests are based on DNA extraction and RT-PCR amplification to detect cffDNA in maternal plasma. The presence of the *RHD* gene sequence in the plasma of a pregnant *RHD* negative woman suggests an *RHD*-positive fetus. Many clinical centres have already established NIPD techniques for *RHD* genotyping (UK, Netherlands and France) (Chiu and Lo, 2011).

1.4.2 Fetal sex determination

Fetal sex determination is used to identify if there is a risk of an X- linked disease, through detection of sequence present on Y chromosome in plasma of pregnant women carrying male fetus (Finning and Chitty, 2008). This is used in X-linked disorders, for example, carrier mothers of serious X-linked conditions like Duchenne muscular dystrophy and Hunter disease (autosomal recessive disorder which primarily affects males), which confer significant morbidity and mortality. Therefore, genetic prenatal diagnosis for women carrying male fetuses is required. Gender determination using cffDNA appears to be reliable from as early as 7 weeks of gestation (Scheffer et al., 2010); this allows women who need invasive procedures to have a CVS at 11 weeks, with knowledge that they are not unnecessarily putting a pregnancy at risk. Furthermore early determination of fetal gender is of use in the clinical management of fetuses at risk of congenital adrenal hyperplasia (CAH), which is a group of genetic

disorders that are inherited in an autosomal recessive fashion and result from an over production of adrenal androgen. Excessive production of androgen has no effect on male external genitalia but varying degrees of virilisation occur in affected female fetuses subsequent to the exposure to high levels of androgens. Virilisation starts as early as 8 weeks of gestation, but administration of dexamethasone to the mother can prevent virilisation by suppressing hypothalamic-pituitary-adrenal axis (Avent and Chitty, 2006). Male pregnancies will not need steroid therapy, while female pregnancies could have steroid prophylaxis for a short period of time prior to CVS at 11 weeks. Maximum effect of dexamethasone starts at the sixth week of gestation. NIPD will allow determination of fetal sex early on in pregnancy, hence allowing dexamethasone treatment to start early and therefore achieving maximum effect.

1.4.3 Single gene disorder

Currently available techniques are unsuitable for diagnosis of X-linked and recessive disorders, as the fetal genes inherited from the mother are swamped by the excess of the mother's own DNA. In recessively inherited conditions, such as cystic fibrosis or thalassemia, if the parents carry different mutations then exclusion of paternal allele from the maternal plasma indicates that the fetus would be unaffected but, if the paternal allele is present, an invasive test is required to determine whether the fetus has inherited the abnormal maternal allele as well. The possibility of increasing scope for NIPD of single gene disorders to include recessive disorders or conditions inherited maternally, was demonstrated by Lo and Chiu (2008) when they used digital PCR to detect small differences in the levels of the mutant and wild type alleles of disease causing genes and to determine whether they were balanced or unbalanced and the fetus thus unaffected or affected. Recently a study by Barrett et al. (2012) has shown the use of digital PCR for NIPD of sickle cell anaemia.

1.4.4 NIPD for chromosomal aneuploidies

NIPD using cffDNA for chromosomal aneuploidies like trisomy 21 has been more difficult because of the high maternal free DNA background. Any method that can increase the relative percentage of cffDNA in the sample would make it easier to distinguish fetal from maternal DNA and hence detect an abnormal concentration of a particular chromosome.

Gene dosage assessment can be obtained with quantitative PCR, which is possible due to the high polymorphisms of STRs. Specific chromosome markers for chromosome 21 identify two peaks of fluorescent activity with a ratio of 1:1, however trisomic samples will produce either trisomic triallelic (three peaks; ratio 1:1:1) or trisomic diallelic (two peaks; ratio; 1:2/ 2:1) results (Cirigliano et al., 1999). Zimmermann et al. (2002) identified that through DNA quantification of a chromosome 21 locus and a reference locus identified a 1.5 fold increase in chromosome 21 DNA sequences, however because a 2-fold difference in DNA template concentrations only constitutes one Ct, conventional RT-PCR assays have shown to be limited to a 1.5 fold discrimination difference. Digital PCR is an alternative strategy that can detect overrepresentation of chromosome 21 with respect to a reference chromosome, with a finer degree of quantitative discrimination (Lo et al., 2007a).

1.5 Methods to enrich cffDNA

Currently, several assay procedures are developed in order to enrich and enhance the fractional concentration of cffDNA or just to distinguish the cffDNA in maternal blood samples. One point of differentiation between cffDNA and cell free maternal DNA is that the former has a shorter size distribution (Li et al., 2004a). Based on this observation, researchers tried to apply a method of size fractionation with the aid of various kits and columns that rely on the inability of large molecular weight DNA to pass through or by retention of low molecular weight DNA in a gel or column (Legler et al., 2007). Main disadvantage of these approaches are: the currently used electrophoretic method is labour intensive and probably prone to contamination. It is unknown if the provided DNA enrichment is satisfactory enough for the prenatal diagnosis of chromosomal aneuploidies (Lo and Chiu, 2008).

Dhallan et al. (2004) reported another approach for cffDNA enrichment in maternal circulation. They proposed that through fixation of maternal nucleated blood cells and the use of formaldehyde, the dilution of fetal DNA could be avoided in maternal plasma. They went on to demonstrate the utility of this approach for NIPD of trisomy 21. There are two speculations about the role of formaldehyde in increased yield of fetal DNA: a) prevention of maternal cell lysis and subsequent reduction of amount of cell free maternal DNA, b) prevention of the degradation of cffDNA by its nuclease inhibitory effect. However Chinnapapagari et al. (2005) and Chung et al. (2005), demonstrated that the formaldehyde addition did not yield a dramatic increase in fetal DNA concentration as previously reported by Dhallan. A possible reason for this discrepancy is that the sample processing time differs between the studies and

it is known that the amount of time spent in the tube affects the concentration of total cell free DNA. Zhang et al. (2008) proposed that the formaldehyde addition will offer a beneficial effect if there is a delay of > 6 hours in sample processing as they demonstrated no maternal blood lysis or released extra maternal free DNA in to plasma within the first six hours.

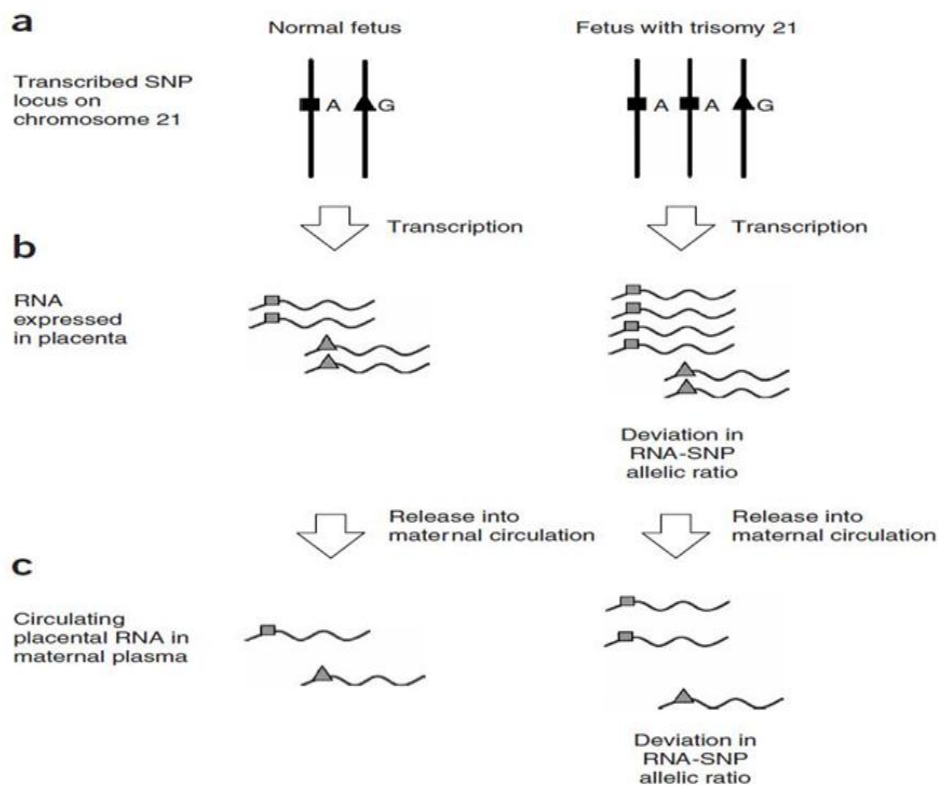
Bischoff (2011) demonstrated that cffDNA is resistant to degradation by DNase, supporting the hypothesis that cffDNA is packaged in membranous bound vesicles that are formed as a result of apoptosis. They described a novel two stage method for enrichment of fetal fragments. The first stage involves treatment of total maternal plasma with DNase. Given that fetal fragments are more stable and likely packaged by membranous bound apoptotic bodies, they hypothesized DNase treatment would deplete the unpackaged maternally derived sequence. The second stage involves a modified whole genome amplification (WGA) protocol designed to amplify smaller fetal fragments.

1.6 Methods to detect fetal aneuploidy by NIPD

1.6.1 RNA single nucleotide polymorphism allelic ratio

Allelic ratio approach is regarded as the most promising single marker approach for NIPD of trisomy 21 (Lo et al., 2007b). To achieve diagnosis of fetal chromosomal aneuploidy from maternal plasma, it would be reasonable to target nucleic acid molecules, which were fetal specific (free from maternal background interference) and then to derive chromosome dosage information from such molecules (Ferguson, 2003). To provide chromosomal dosage information the circulating placental mRNA has to be transcribed from chromosome of interest. When SNP is transcribed the fetus with trisomy 21 will have an extra copy of the gene. The gene is then expressed in the placental tissue and the ratio of the two RNA alleles in the trisomy 21 placenta will differ from normal placenta when the transcripts are released in to the maternal circulation. The difference in allelic ratio is reflected in the abundance of the transcripts, that is the level of circulating placental RNA will be greater in trisomy 21 samples (Figure 1). This method is also applicable to the prenatal diagnosis of other fetal chromosomal aneuploidies like trisomy 18 and 13. However the dependence on genetic polymorphism limits the use of these approaches to heterozygous fetuses.

Figure 1: The RNA-SNP allelic ratio method of identifying the difference between normal and DS fetuses who are heterozygous at the SNP locus of PLAC4, which is transcribed by chromosome 21 and expressed from the placenta. PCR amplification was used to determine the ratio of SNPs in PLAC4. As the ratio of two alleles in a heterozygous euploid fetus would be 1:1 whereas in a trisomic fetus the ratio would be either 1:2 or 2:1. Using maternal plasma this ratio could be determined and the number of copies of chromosome 21 could be identified noninvasively. (Lo et al., 2007c) *Nature Med.* 13: 218-223



1.7 Single molecule counting methods

The urgent need for the widespread application of NIPD for the detection of trisomy 21 has created strong interest in rapid and accurate single molecule counting methods (digital PCR and next generation sequencing).

Single molecule counting methods could be used in routine clinical diagnosis; these methods will be polymorphism independent, and will detect trisomy 21 cases based on the presence per se of an elevated amount of chromosome 21 sequences in maternal blood. The main disadvantage of these approaches is that they require the counting of an extremely large number of molecules for markers that are not fetal-specific, and their use demands expensive equipment, reagents, and in addition, complex bioinformatics methods.

1.7.1 Digital PCR

Recent reports have indicated that digital PCR, a method that was initially applied for determination of the allelic frequencies of oncogenic alterations in samples from patients with cancer, could be a valuable new tool in NIPD of trisomy 21 (Zimmermann et al., 2008). Digital PCR is a highly sensitive technique that uses dilutions to isolate single template DNA molecules to be amplified in order to detect very small differences in chromosome ratios. In this method fetal derived DNA is not specifically distinguished from maternal DNA instead the technique provides a measure of total (fetal and maternal) dosage of particular chromosome relative to another reference chromosome. A significant barrier for using digital PCR is the small fraction of cffDNA in maternal plasma, so this technique requires prior enrichment of cffDNA to achieve high accuracy. The analytical precision of digital PCR is superior to that of RT-PCR because template molecule quantification does not rely on response relationships between reporter dye and nucleic acid concentration.

To test whether digital PCR is precise enough to detect fetal chromosomal aneuploidies in maternal plasma, Lo et al. (2007a) assessed first whether digital PCR could measure the allelic ratio of PLAC4 mRNA in maternal plasma, thereby distinguishing trisomy 21 from euploid fetuses. They refer to this as the digital RNA SNP method. Then they evaluate whether the increased precision of digital PCR would allow the detection of fetal chromosomal aneuploidies without depending on genetic polymorphisms, and they referred to this approach as digital relative chromosome dosage (RCD) analysis.

1.7.2 Next generation sequencing

The principle behind shotgun sequencing is to sequence millions of short DNA fragments at the same time. The numbers of sequences from different chromosomes are then compared. If a certain sequence is slightly over-represented this may indicate aneuploidy or chromosomal imbalances. The main advantage is that it is polymorphism-independent and it could be used in all pregnancies. While the disadvantages are that the approach is prohibitively expensive, slow and requires large amount of data processing and interpretation. Fan et al. (2008), Chiu et al. (2010) and (Chen et al., 2011) reported the successful use of this method for the detection of trisomy 21 at gestational ages as early as 14th week. Nevertheless, all these techniques are still new and more clinical trials need to be conducted in order to assess their clinical effectiveness as a substitute for traditional invasive diagnostic testing.

1.8 Ethics

Prior to NIPD, prenatal testing was already associated with high termination rates. But unlike these prior tests, NIPD provides more than an estimated likelihood that a fetus will be affected by a certain condition and can be performed from the middle of the first trimester of the pregnancy. It is essential to realize that there are significant ethical issues that are associated with NIPD. Fetal sex determination as early as seven weeks of pregnancy (using NIPD) carries a number of socio-ethical implications, such as the promoting the selective termination of fetuses according to gender (Marteau and Chitty, 2006). Another issue with NIPD for fetal sex determination is that women might seek prenatal diagnosis to include paternity testing.

An additional concern deals with ensuring that women retain their right to informed permission for testing. The worry is that with the risk of miscarriage removed, health care providers may fail to adequately inform the patients of the remaining implication of test outcomes, including learning prior to birth that they are pregnant with a child affected with a genetic disease, and thus patients may be faced with serious decisions that they were not prepared for. Increasing terminations of pregnancies, options for regulation, and non-medical uses such as sex selection, are also raised as potential ethical concerns (van den Heuvel et al., 2008). A serious disadvantage of early NIPD is that it may increase the burden of choice for women, since abnormal fetuses will be identified that would have miscarried spontaneously later in pregnancy (Wright and Burton, 2009).

Prenatal diagnosis will become safer for many women by removing the risks associated with invasive tests. This may in turn compromise informed decision making since, by removing the need to discuss the miscarriage risk, the

decision maker may fail to adequately consider the remaining implications of test outcomes (van den Heuvel et al., 2008). Excellent communication with women and health professionals is essential to understanding the implications of the simple, safe blood tests that they will be offered to detect trisomy 21 as well as other conditions.

2 Chapter Two: Materials and methods

2.1 Sample collection and processing

In this study, female genomic DNA (G1521) (Promega, Southampton) was used as a template. Primers for amplification as shown in Appendix A were designed by Alice Bruson (a visiting PhD student from Università degli di Padova, Italy) using the Primer3 software (<http://fodo.wi.mit.edu/primer3>). Following primer design the sequences were subsequently BLASTed to ensure primer specificity for the chromosome of interest using Primer-BLAST software. The internal and external primers (Eurofins MWG Operon) (www.eurofinsdns.com, Ebersberg) used in this study are shown in Table 1. The designed probe (Eurofins MWG Operon) had an attached 5'FAM reporter label and a black hole quencher 1 (BHQ1) quencher dye attached at the 3' end.

The **TaqMan Probe** (Applied Biosystems) is designed as a high-energy dye termed a Reporter at the 5' end, and a low-energy molecule termed a Quencher at the 3' end. When this probe is intact and excited by a light source, the reporter dye's emission would be suppressed by the Quencher dye as a result of close proximity of the dyes. When the probe is cleaved by the 5' nuclease activity of the enzyme, the distance between the Reporter and Quencher increases, causing the transfer of energy to stop. The fluorescent emission of reporter increases and the quencher decreases.

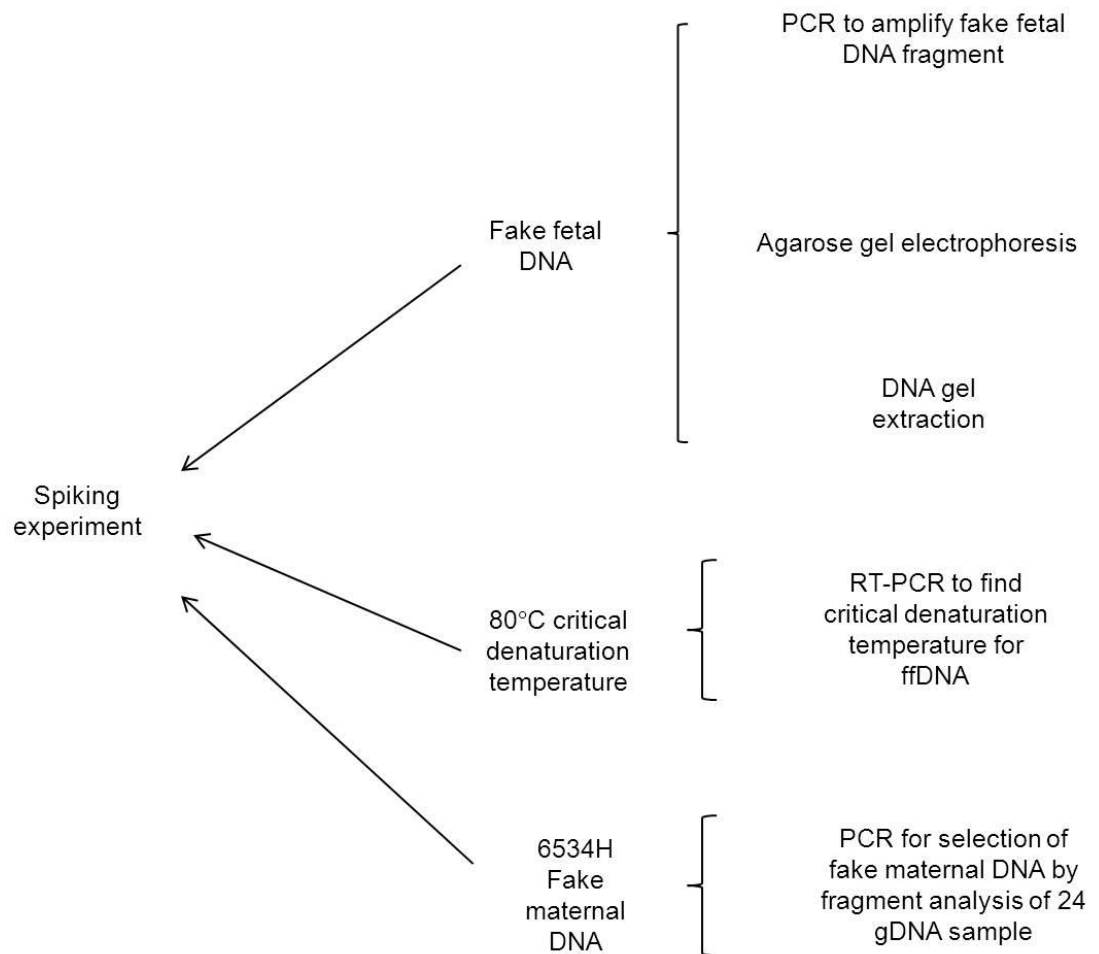
All primers and probe were HPLC (high performance liquid chromatography) purified. The oligonucleotide synthesis report provided with the primers contained other important information including the calculated annealing temperature for each primer (see Table 1). The chosen fragment on chromosome 21 contains a CA repeat STR identified as D21S1890.

Genomic DNA for fragment analysis (to act as fake maternal DNA) was extracted from buffy coat; twenty four genomic DNA samples were used to provide various DNA templates. These samples were collected from random donors from National Health Service Blood and Transplant (NHSBT) (Filton, Bristol, UK). The DNA was extracted using QIAamp DNA blood mini kit (Qiagen Ltd, UK) by personnel in Neil Avent's Laboratory, Plymouth University.

Table 1: The primers and probe sequences for D21S1890.

Oligo name	Sequence (5' – 3')	GC-Content	Tm (°C)
D21S1890 Internal Forward	TCGCCCCGAGGGTCTGA	68.8%	56.9°C
D21S1890 Internal Reverse	AAACCAACTGACTCCCAAACAAC	43.5%	58.9°C
D21S1890 Internal Forward	TCGCCCCGAGGGTCTGA 5' labelled with FAM	68.8%	56.9°C
D21S1890 External Forward	GGAGAAACGAGGATGAGCTTC	52.4%	59.8°C
D21S1890 External Reverse	TATCCCCCGTATTCTTGGT	45%	55.3°C
D21S1890 External Forward	GGAGAAACGAGGATGAGCTTC 5' labelled with FAM	52.4%	59.8°C
D21S1890 Probe	AGATTTCCTCAATCGCCA 5' labelled with FAM 3' labelled with BHQ1	47.1%	50.4°C

Figure 2: overview of the experimental protocols used.



2.2 Methods

2.3 Polymerase chain reaction (PCR)

2.3.1 PCR to amplify fake fetal DNA fragment

PCR was used to generate a fake fetal DNA fragment using a Veriti 96-well Thermal Cycler (Applied Biosystems, California, USA). The conditions for the PCR were as follows; 95 °C for 10 minutes followed by 40 cycles of 95 °C for 30 seconds, 56 °C for 30 seconds, then 72 °C for 40 seconds, followed by a final extension of 72 °C for 7 minutes. This final elongation step ensured that any remaining single stranded DNA was fully extended. Female genomic DNA was used as template at a final concentration of 4 ng/μl and GenTaq Master Mix was used at a final 1x concentration. Unlabelled D21S1890 external forward and reverse primers were used both at a final concentration of 200 nM. The PCR reaction volume was 50 μl and this was replicated ten times to give a total reaction volume of 500 μl.

2.3.1.1 Agarose gel electrophoresis

PCR amplicons were analysed using agarose gel electrophoresis. The samples were run on a 2% agarose gel, 1xTAE (40 mM Tris –acetate 1 mM EDTA and acetic acid) (Sigma- Aldrich, UK) and GelRed (Biotium) (1:10.000 dilution) as DNA gel stain in order to view the bands of DNA. 10 µl of marker (Ranger 100 bp DNA ladder, Norgen) was run alongside samples, 100 µl of sample mixed with 10 µl of DNA loading buffer [0.36% (w/v) Orange G, 0.47M Tris-HCl (pH 8, 43% (v/v) glycerol]. Thus total of 110 µl (100 µl DNA sample + 10 µl loading dye) was added to each subsequent well. Electrophoresis was performed at 110 Volts for about 60 minutes. Following the run, the gel was removed and placed in the EC3 imaging system (UVP BioImaging System, Cambridge, UK) and results were analysed using the Launch Vision WorksLS program (Chemi Doc 410).

2.3.1.2 DNA gel extraction

The fragments were excised from the agarose gel using a sharp scalpel and then weighed. DNA fragments were extracted and purified according to manufacturer's instructions in the QIAquick gel extraction kit (Qiagen). Briefly, three volumes of buffer QG were added to 1 volume of gel. Following a 10 minute incubation period at 50 °C, one gel volume of isopropanol was added to each sample and mixed to increase the yield of DNA fragment. DNA samples were added to a QIAquick spin column in a collection tube and following one minute of centrifugation the flow through was discarded. Afterward, the sample was washed by adding 0.75 ml of buffer PE to the QIAquick column and centrifuged for one minute. Flow through was discarded again and re-centrifuged for an additional minute to remove residual ethanol from column. Final step was performed by adding 50 µl of buffer EB to the column and centrifuged for one minute (after placing QIAquick column into a clean 1.5 ml microcentrifuge tube). Once DNA had been extracted it was quantified using the Nano Vue Plus spectrophotometer.

2.3.1.3 Assessment of DNA quality and quantity

The NanoVue Plus Spectrophotometer (GE Healthcare, Little Chalfont, UK) was used to measure DNA concentration and quality of each sample. The optical density was assessed at 260 nm and 280 nm. A DNA quality control threshold was set with an A260/A280 ratio of between 1.6 and 1.95. The extracted DNA was stored at -20°C.

2.3.2 PCR for selection of fake maternal DNA

PCR was conducted using Veriti 96-Well Thermal Cycler (Applied Biosystems) with 5'FAM labelled D21S1890 external forward and non-labelled external reverse primers both at a final concentration of 400 nM. Cycling conditions were same as (Method 2.4.1) but with thirty five cycles instead of forty. Using AmpliTaq Gold (Applied Biosystems) enzyme at a final concentration of 1.25Unit/reaction, dNTPs 200 µM, MgCl₂ 2 mM. Purified DNA of twenty four different genomic DNA from buffy coat samples were used as templates at a final concentration of 4 ng/µl. Twenty six reaction tubes were prepared in total containing a 25 µl reaction volume in each: twenty four tubes containing template DNA (from buffy coat), one tube contains female genomic DNA, and one tube with water as a negative control. 10 µl of PCR products were run out on a 2% agarose gel at 100 volts for one hour.

Thereafter, Dr Michele Kiernan carried out a sequence analysis on 2 µl from the remaining PCR samples using ABI Genetic Analyser 3130 (Applied Biosystems, Warrington, UK). The information obtained was analysed using Peak ScannerTM fragment analysis Software Version 1.0.

AmpliTaq Gold is a thermostable DNA polymerase designed to improve the amplification of templates by lowering the nonspecific background and increasing the amplification of required specific products.

2.3.3 Performing PCR with internal primers for fake fetal and fake maternal DNA fragment

PCR using D21S1890 5'FAM labelled internal forward and non-labelled internal reverse primers, both at a final concentration of 400 nM, was carried out using a Veriti 96-Well Thermal Cycler (Applied Biosystems). Fake maternal DNA (6534H from buffy coat) and fake fetal DNA (from female genomic DNA) were used as templates at a final concentration of 4 ng/μl and 0.4 ng/μl respectively, and TaqMan Universal PCR Master Mix (Applied Biosystems) at 1x concentration. Making up Master Mix to (20 μl/tube) and 5 μl from each template plus one tube of water as a negative control. Running program was as follows; 50 °C for two minutes, 95 °C for ten minute, 50 °C for one minute followed by 45 cycles of 95 °C for fifteen seconds and 56 °C for one minute.

Moreover we have used Enhancer with AmpliTaq Gold enzyme. The Master Taq Kit contains recombinant Taq DNA polymerase purified from *Escherichia coli* DH1, a 10x Taq Buffer with 15 mM MgCl₂, a separate 25 mM Magnesium solution, and the special 5xTaqMaster PCR enhancer to help with the performance of sensitive and sophisticated PCR applications on difficult template DNA.

2.3.4 Real-Time PCR (RT-PCR) to find critical denaturation temperature of fake fetal DNA

RT-PCR was used to find the critical denaturation temperature using D21S1890 internal forward and reverse primers, different concentrations of primers and probes were used as shown in Table 2. PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) at 1x concentration and made up to solutions that allowed 20 µl of mastermix solution to be added to each reaction. Female genomic DNA and fake fetal DNA were used as templates at a final concentration of 4 ng/µl and 0.004 ng/µl respectively. Water was used as a negative control, DNA samples were all in triplicate. 5 µl of each template was used. The cycle sequence was run on the StepOnePlus RT-PCR (Applied Biosystems) as follows: 50 °C for two minute, 95 °C for ten minutes, and 50 °C for one minute, followed by 45 cycles of 95 °C for fifteen seconds and 56 °C for one minute. Series of RT-PCR runs were conducted altering the denaturation temperature for each; they were conducted at (84°C - 83°C - 82°C -81°C- 81.8°C -81.6°C -81.4°C -81.2°C -80°C). The threshold cycle was determined using StepOne Software (v2.1) automatically.

Table 2: Primer and probe concentrations for RT-PCR.

Attempts	Primer Mix	Probe
Attempt A	200 nM	100 nM
Attempt B	300 nM	100 nM
Attempt C	200 nM	50 nM
Attempt D	300 nM	50 nM

2.3.5 SYBR Green RT-PCR

SYBR Green dye is the most commonly used dye for non-specific detection. It is a double-stranded DNA intercalating dye, that fluoresces once bound to the DNA. A pair of specific primers is required to amplify the target with this chemistry. The amount of dye incorporated is proportional to the amount of the generated target. The inconvenience of this technique is that SYBR Green I will bind to any amplified dsDNA. Consequently, primer dimers or unspecific products introduce a bias in the quantification. However, it is still possible to check out the specificity of the system by running a melt curve analysis at the end of PCR run. The principle is that every product has a different dissociation temperature, depending on the size and base composition, so it is still possible to check the number of products amplified.

Instead of using fluorescent probe, 1x SYBR Green mastermix (Applied Biosystems) was added to forward and reverse D21S1890 primers both at final concentration of 200 nM and made up total mix that allowed 20 µl of master mix solution to be added to each reaction. Melt curve analysis was carried out using gradient options on StepOnePlus software to run two temperatures at the same time one at 95 °C as a positive control and the other at 80 °C (critical denaturation temperature). As SYBR Green binds to any double stranded DNA the amount of fluorescence indicates the amount of dsDNA present. As the DNA is heated to the denaturation temperature the dsDNA melts to single stranded DNA and the denaturation temperature is detected by a loss of fluorescence, the midpoint of this transition is known as melting temperature of the sample and is characteristic of a given DNA sequence. Therefore melt

curves are useful for determining the specificity of a PCR reaction, as any nonspecific amplification products will have a different melt curve profile than the target sequence (Ririe et al., 1997). The templates were 6534H genomic DNA (fake maternal DNA) at a final concentration of 4 ng/μl, fake fetal DNA at 0.4 ng/μl and spike sample (90% fake maternal DNA and 10% fake fetal DNA). Plus water as a negative control. Samples were all run in triplicate.

2.3.6 PCR for cloning experiment

PCR using D21S1890 external forward and reverse primers was carried out using a Veriti 96-Well Thermal Cycler (Applied Biosystems). The primers were used at a final concentration of 200 nM. Fake fetal and fake maternal DNA were used as templates at a final concentration of 0.4 ng/μl and 4 ng/μl respectively. BioMix Master Mix (Bioline, Ltd UK) was used at 1x final concentration. Thus two samples were prepared each with 20 μl of Master Mix, one with 5 μl of fake fetal DNA and the other with 5 μl of fake maternal DNA. The Cycle sequence was as follows; 95 °C for 10 minutes followed by 40 cycles of 95 °C for 30 seconds , 56 °C for 30 seconds, then 72 °C for 60 seconds, followed by a final extension of 72 °C for 20 minutes. PCR products were cloned into pCR[®]-4 TOPO[®] (Invitrogen[™]), using the TOPO TA cloning method according to the manufacturer's protocol (Method 2.8.4).

2.4 Whole genome amplification of fake maternal DNA (6534H)

REPLI-g UltraFast Mini Kit (Qiagen) was used to amplify genomic DNA (sample 6534H) according to the manufacturer's instructions. The method is based on Multiple Displacement Amplification (MDA) technology, which carries out isothermal genome amplification utilizing a uniquely processive DNA polymerase capable of replicating 100 kb without dissociating from the genomic DNA template. The sample is lysed and the DNA is denatured by adding denaturation buffer. After denaturation has been stopped with addition of neutralization buffer, a Master Mix, containing reaction buffer and REPLI-g UltraFast DNA Polymerase, was added. The isothermal amplification reaction proceeds for one and a half hours at 30 °C.

The first step was to prepare Buffer D1 (denaturation buffer) and Buffer N1 (neutralization buffer), Buffer D1 was made by adding 2.5 µl of reconstituted buffer DLB and 7.5 µl of nuclease free water. Buffer N1 was made from 4 µl of stop solution and 36 µl of nuclease free water. Then 1 µl of 6534H genomic DNA was added into a microcentrifuge tube, 1 µl buffer D1 added to the sample and mixed by vortexing and centrifuging briefly and then the sample incubated at room temperature for three minutes. After that 2 µl buffer N1 added to the sample and vortexed again. Master Mix was made from 15 µl REPLI-g UltraFast reaction buffer and 1 µl REPLI-g UltraFast DNA polymerase, the total 16 µl was added to 4 µl of denatured DNA and incubated at 30 °C for one and half hours. Heating of sample was required to inactivate REPLI-g UltraFast DNA polymerase for that the sample was heated on a hot block of 65 °C for three

minutes. Concentration was calculated from absorbance reading 893.5 ng/μl, the A260/A280 ratio was 1.619, and the A260/A230 ratio was 1.742. The extracted DNA was stored at -20 °C.

2.5 Spike Experiment

The purpose of spiking experiment was to amplify the fake fetal DNA fragment without amplifying genomic DNA. Spike experiments were performed using fake fetal fragment (amplified from female genomic DNA, Promega) and fake maternal DNA (6534H genomic DNA from buffy coat) with D21S1890 internal forward and reverse primers at a final concentration of 200 nM and TaqMan probe 100 nM.

Two RT-PCR reactions were performed one with a denaturation temperature of 95 °C as a positive control and a second reaction performed with a critical denaturation temperature of 80 °C. At the same time as setting up the spiking experiment for RT-PCR we have set identical runs for Veriti (endpoint PCR) with the final volume of 50 µl per each reaction (for fragment analysis and acrylamide gel), instead of using labelled probe, 5'FAM labelled D21S1890 internal primer was used at a final concentration of 200 nM. Fake fetal fragment and 6534H genomic DNA were used in order to establish standard curve, this was carried out by making different dilutions of genomic DNA which was used as standard curve at a denaturation temperature of 95 °C (20 ng/µl, 2 ng/µl, 0.2 ng/µl and 0.02 ng/µl) and different dilutions of fake fetal DNA (2 ng/µl, 0.2 ng/µl, 0.02 ng/µl and 0.002 ng/µl) were used as standard curve at critical denaturation temperature of 80 °C. Therefore four plates were run at two different temperatures using RT-PCR StepOnePlus and endpoint PCR.

The spike samples were made of mixture of fake fetal and fake maternal DNA in two different concentrations; spike A was made of 90% of fake maternal DNA and the remaining 10% being fake fetal DNA. Spike B was made of 95% of fake maternal DNA and the remaining 5% of fake fetal DNA as shown in Table 3.

Genomic A and B were used as templates, genomic A (90% genomic DNA and 10% water), genomic B (95% genomic DNA and 5% water). Fetal A and B also used as templates, fetal A (90% water and 10% fake fetal fragment), fetal B (95% water and 5% fake fetal fragment). Water was used as negative control (NTC) and each sample was run in triplicate as shown in plate layout (Table 4 and Table 5). The PCR products with internal D21S1890 FAM labelled forward primer (endpoint PCR) were run out on an agarose gel to check product size, on an acrylamide gel, and 1-2 μ l of remaining PCR product was used for fragment analysis on genetic analyser.

Table 3: The composition of spike samples.

Categories	Amount of fetal DNA per well	Amount of genomic DNA per well
Spike A	10 ng	90 ng
Spike B	5 ng	95 ng

Table 4: The table displays the plate layout of the spike experiment at a critical denaturation temperature (Td) of 95 °C (as a positive control plate). Fake maternal DNA (6534H) at four different dilutions: [S1 (20 ng/μl), S2 (2 ng/μl), S3 (0.2 ng/μl), S4 (0.02 ng/μl)] were used as a standard curve (STD). spike A (90% fake maternal DNA + 10% fake fetal DNA), spike B (95% fake maternal DNA + 5% fake fetal DNA). Genomic DNA A (fake maternal DNA) = 90% gDNA + 10% water, genomic DNA B = 95% gDNA + 5% water. Fake fetal DNA A (FFDNA. A) = 90% water + 10% fetal DNA, fake fetal DNA B= 95% water + 5% fetal DNA.

	1	2	3	4	5	6	7	8	9	10	11	12
A	6534H 20ng/μl STD	6534H 20ng/μl STD	6534H 20ng/μl STD	6534H 2ng/μl STD	6534H 2ng/μl STD	6534H 2ng/μl STD	6534H 0.2ng/μl STD	6534H 0.2ng/μl STD	6534H 0.2ng/μl STD	6534H 0.02ng/μl STD	6534H 0.02ng/μl STD	6534H 0.02ng/μl STD
B	Spike. A 90%-10%	Spike. A 90-10%	Spike. A 90%-10%	Spike. B 95%-5%	Spike. B 95%-5%	Spike. B 95%-5%	WATER NTC	WATER NTC	WATER NTC			
C	gDNA. A 20 ng/μl	gDNA. A 20 ng/μl	gDNA. A 20 ng/μl	gDNA. B 20 ng/μl	gDNA. B 20ng/μl	gDNA. B 20 ng/μl						
D	FFDNA A 2ng/μl	FF DNA A 2ng/μl	FFDNA A 2ng/μl	FFDNA B 2ng/μl	FFDNA B 2ng/μl	FFDNA B 2ng/μl						

Table 5: The table displays the plate layout of the spike experiment at a critical Td of 80°C. Fake fetal DNA at four different dilutions: [S2 (2 ng/μl), S3 (0.2 ng/μl), S4 (0.02 ng/μl), S5 (0.002 ng/μl)] were used as a standard curve (STD). Spike A (90% fake maternal DNA + 10% fake fetal DNA), spike B (95% fake maternal DNA + 5% fake fetal DNA). Genomic DNA A (fake maternal DNA) = 90% gDNA + 10% water, genomic DNA B = 95% gDNA + 5% water. Fake fetal DNA A (FFDNA. A) = 90% water + 10% fetal DNA, fake fetal DNA B= 95% water + 5% fetal DNA.

	1	2	3	4	5	6	7	8	9	10	11	12
A	FFDNA 2ng/μl STD	FFDNA 2ng/μl STD	FFDNA 2ng/μl STD	FFDNA 0.2ng/μl STD	FFDNA 0.2ng/μl STD	FFDNA 0.2ng/μl STD	FFDNA 0.02ng/μl STD	FFDNA 0.02ng/μl STD	FFDNA 0.02ng/μl STD	FFDNA 0.002ng/μl STD	FFDNA 0.002ng/μl STD	FFDNA 0.002ng/μl STD
B	Spike. A 90%-10%	Spike. A 90%-10%	Spike. A 90%-10%	Spike.B 95%-5%	Spike.B 95%-5%	Spike.B 95%-5%	WATER NTC	WATER NTC	WATER NTC			
C	gDNA.A 20ng/μl	gDNA.A 20ng/μl	gDNA.A 20ng/μl	gDNA.B 20ng/μl	gDNA. B 20ng/μl	gDNA.B 20ng/μl						
D	FFDNA A 2ng/μl	FFDNA A 2ng/μl	FFDNA A 2ng/μl	FFDNA B 2ng/μl	FFDNA B 2ng/μl	FFDNA B 2ng/μl						

2.6 Polyacrylamide Gel Electrophoresis

Non-denaturing polyacrylamide gel electrophoresis was used for separation and purification of fragments of double stranded DNA. A mini-PROTEAN TBE Precast Gel (10% acrylamide) (Bio-Rad Laboratories) was used. The gel was run using a mini PROTEAN Tetra System. Loading dye was made from [12.5 mM Tris (pH 8), 30% (v/v) glycerol, 0.25% (w/v) bromophenol blue]. 3 µl of loading dye was added to 15 µl of PCR product generated from fake maternal DNA (samples A and B), fake fetal DNA (samples A and B) and admixes of fake maternal and fetal DNA (spike A and spike B) at denaturation temperatures of 95 °C and 80 °C. 18 µl of these samples were added (15 µl PCR sample + 3 µl loading dye) to the gel along with 12 µl of marker (Ranger 100bp DNA ladder, Norgen). The gel was run in 1x TBE (10XTBE (Bio-Rad laboratories. Tris, Boric Acid and EDTA buffer) for two and half hours at sixty volts then transferred to a gel tray with 20 ml 1x TBE and 7.5 µl GelRed (Biotium) 1:10.000 dilution as DNA gel stain. This was transferred to a gyro-rocker and rocked at room temperature for sixty minutes. And then the gel was placed in the EC3 imaging system (UVP Bio Imaging System, Cambridge, UK) and results were analysed using Launch vision WorksLS program (Chemi Doc 410).

2.7 Cloning experiment

Experimental outline

- ❖ Preparing Luria Bertani media and agar
- ❖ Producing PCR Product
- ❖ Cleaning of PCR product
- ❖ TOPO[®] Cloning reaction (mixing PCR product and pCR[®] 4-TOPO[®])
- ❖ Transforming into One Shot[®] Mach 1[™]- T1[®] Chemically Competent *E. coli* cells
- ❖ Selecting and analysing colonies
- ❖ Performing colony PCR
- ❖ Isolating plasmid DNA
- ❖ Digestion of plasmid DNA
- ❖ Sequencing of plasmids

2.7.1 Cleaning of PCR product

After performing a PCR for cloning reaction (Method 2.4.6), QIAquick Gel Extraction Kit (Qiagen) was used for cleaning the DNA from enzymatic reactions according to the manufacturer's instructions. Through adding three volumes of Buffer QG and one volume of isopropanol to the PCR reaction, then for binding DNA the samples was added to QIAquick column and centrifuged for one minute. For washing step, 0.75 ml of Buffer PE was added to QIAquick column and centrifuged for one minute. To completely remove residual ethanol from the column, the flow-through was discarded and centrifuged for additional one minute. Final step was performed by adding 50 μ l of Buffer EB to the column (after placing QIAquick column into a clean 1.5 ml microcentrifuge tube) and the column was centrifuged for one minute.

Concentrations were calculated from absorbance reading using Nano Vue Plus spectrophotometer (GE Healthcare):

Female genomic DNA: 20.5 ng/ μ l, the A260/A280 ratio was 1.708, and the A260/A230 ratio was 0.061.

Fake maternal DNA: 20.0 ng/ μ l with A260/A280 ratio of 1.674, and the A260/A230 ratio was 0.325.

2.7.2 Luria Bertani Media (LB Media)

The LB media was prepared by dissolving 10 g of LB broth (Sigma) in 500 ml of distilled water. The solution was autoclaved and stored at room temperature until required.

2.7.3 Luria Bertani Agar (LB plates)

The LB plates were prepared by dissolving 12 g of Bacteriological Agar powder (Sigma) in one litre of distilled water. The solution was sterilized by autoclaving and cooled to 50 °C prior to the addition of the appropriate antibiotics at the required concentration. The selective plates used in this study included ampicillin ready-made solution (Sigma) at a concentration of 50-100 µg/ml.

For making twenty plates of LB agar with ampicillin and X-gal; 300 µl of ampicillin were added to 300 ml of LB agar under aseptic condition, and then thin layer (5 mm) of LB Agar poured in to each plate (swirl plate in a circular motion to distribute agar on bottom completely).

For Blue/White Colony Screening 5-bromo -4-chloro-indolyl-β-D-galactopyranoside (5 mg substrate per tablet) (X-Gal). X-Gal was made from dissolving two tablets into 500 µl of Dimethylformamide (DMF); 40 µl of X-Gal applied to top of agar, and spread over the entire surface of the plate under aseptic condition.

2.7.4 Performing the TOPO[®] cloning Reaction

The freshly purified PCR Product was cloned into the vector pCR[®]-4TOPO[®] (Invitrogen). To perform the TOPO Cloning reaction 1 µl of fresh PCR product, 1 µl of salt solution (1.2 M NaCl, 0.06 M MgCl₂), 1 µl of TOPO[®] vector and 2 µl of water were added into microcentrifuge tube mixed gently and incubated for five minutes at room temperature.

2.7.5 Transformation of DNA into Mach 1™- T1R competent *E. coli* cells

Transformation is the introduction of a plasmid into a competent cell. A competent cell is a cell that is chemically treated to allow its membrane to be permeated by plasmids. The plasmid will be replicated in the bacteria, which will copy the DNA fragment of interest. Often the plasmid carries a gene that can make the bacteria resistant to an antibiotic. Only the bacteria that carry the plasmid will grow in the presence of that antibiotic.

After performing TOPO® Cloning reaction, 2 µl from total reaction volume (5 µl) was added into a vial of One Shot® Mach 1™- T1R Chemically competent *E. coli* cells. After 30 minutes incubation on ice, cells were heat shocked for 30 seconds at 42 °C and then incubated for an hour in S.O.C medium (2% w/v Tryptone, 0.5% w/v Yeast Extract, 10 mM NaCl, 2.5 mM, KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose; provided with the kit) at 37 °C. After the incubation, 150 µl was transferred to the centre of an agar plate containing appropriate antibiotic, and a sterile spreader was used to spread the solution over the entire surface of the plate. The plates were stored at room temperature until the liquid had been absorbed, then the plates were inverted and incubated overnight at 37 °C.

2.7.6 Colony PCR

PCR was done to analyse positive transformants. Colony PCR was performed for female genomic DNA by picking five white colonies from plate and one blue colony, each one of these colonies were added to 300 µl of nuclease free water and then heated on heat block at 95 °C for five minutes. M13 forward (5'-GTAAAACGACGGCCAGT-3') and reverse (5'-AACAGCTATGACATG-3') primers were used at a final concentration of 100 nM. BioMix Master Mix (Bioline) was used at 1x final concentration. Therefore six reaction tubes were prepared (5 samples of white colony, 1 sample blue colony). The cycle sequence was run on the Veriti 96 well thermal cycler (Applied Biosystems) as follows; 95 °C for 5 minutes followed by 40 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, then 72 °C for 60 seconds. Succeeding the 40 cycles, there was a final elongation step at 72 °C for 5 minutes. PCR samples were run out using agarose gel electrophoresis.

2.7.7 Isolation of plasmid DNA

For female genomic DNA sample, five colonies were picked up and grown in liquid selective LB medium (5 universal bottles each containing 10 ml of LB medium + 100 µl of ampicillin) for 16 hours at 37 °C, with vigorous shaking. The plasmids containing the insert had to be isolated from the bacterial cells. Therefore plasmid DNA purification was done using (QIAprep spin Miniprep Kit) (Qiagen) according to the manufacturer's instructions. 2 ml overnight culture of *E. coli* in LB medium was transferred to a microcentrifuge tube and centrifuged for one minute. The supernatant was discarded; pelleted bacterial cells were resuspended in 250 µl buffer P1 and transferred to a microcentrifuge tube. Then 250 µl of buffer P2 was added and mixed thoroughly by inverting the tube 4-6 times. 350 µl of buffer N3 was added and mixed immediately, but thoroughly, by inverting the tube 4-6 times. After that the mixture was centrifuged for ten minutes at 13,000 rpm (17,900xg). The supernatants applied to the QIAprep spin column by decanting and centrifuged for sixty seconds. QIAprep spin column was washed by adding 0.75 ml of buffer PE and centrifuged for sixty seconds. To remove residual wash buffer, flow through was discarded and the column centrifuged for an additional one minute. To elute DNA the QIAprep column placed in clean 1.5 ml microcentrifuge and 50 µl of buffer EB was added to the centre of QIAprep spin column and centrifuged for one minute. Isolated plasmids were screened for an insert by digestion with an *EcoRI* restriction enzyme (Promega, Southampton) at 37 °C for one hour.

2.7.8 Digestion of plasmid DNA

Restriction enzymes are enzymes that recognize short DNA sequences. They cleave double stranded DNA at specific sites within or adjacent to their recognition sequences. Recognition sequence for *EcoRI* is 5'...GAATT C...3', 3'...C TTAA G...5'. One unit is defined as the amount of enzyme required to completely digest 1 µg of DNA in one hour at 37 °C in 50 µl assay buffer containing Acetylated Bovine Serum Albumin (BSA) added to a final concentration of 0.1 mg/ml. Thus 1 µl DNA (after plasmid DNA purification), 1 µl of buffer H [1x buffer H supplied with the enzyme has composition of 90 mM Tris-HCl (pH 7.5), 50 mM NaCl and 10 mM MgCl₂ at 37 °C], 0.25 µl of restriction enzyme (three units) and 0.1 µl of BSA was added to 7 µl of nuclease free water. The reaction mixture was centrifuged and then incubated at 37 °C for one hour. When digestion was completed reaction volume was mixed with 2 µl of loading dye [12.5 mM Tris (pH 8), 30% (v/v) glycerol, 0.25% (w/v) bromophenol blue] and run on the 10% acrylamide gel for two and half hours at sixty volts. And then the gel was placed in the EC3 imaging system (UVP Bio Imaging System, Cambridge, UK) and results were analysed using Launch vision WorksLS program (Chemi Doc 410).

2.7.9 Sequencing

The plasmid DNA from the starting PCR template of female genomic DNA were sent for sequencing by (Eurofins MWG Sequencing) using M13 forward and reverse primers. Each sample was diluted to the final concentration of 50 ng/μl in minimum volume of 15 μl.

3 Chapter Three: Results

3.1 Amplification of fake fetal DNA fragment

A PCR was carried out for the amplification of the 229 bp fake fetal DNA fragment from female genomic DNA using the D21S1890 external forward and reverse primers. This was done by using PCR and electrophoresis followed by gel extraction. Figure 3 shows five thick bands fluorescing strongly under UV light between 200 bp and 300 bp (expected size 229 bp) according to the DNA ladder. The product produced will be referred from here onwards as fake fetal DNA fragment. After gel extraction of these bands the fragment DNA recovered was quantified using a NanoVue Plus Spectrophotometer. Concentrations were calculated from absorbance reading 14.1ng/μl, the A260/A280 ratio was 2.178, and the A260/A230 ratio was 0.016. Hence this sample was regarded as fake fetal DNA fragment in this study.

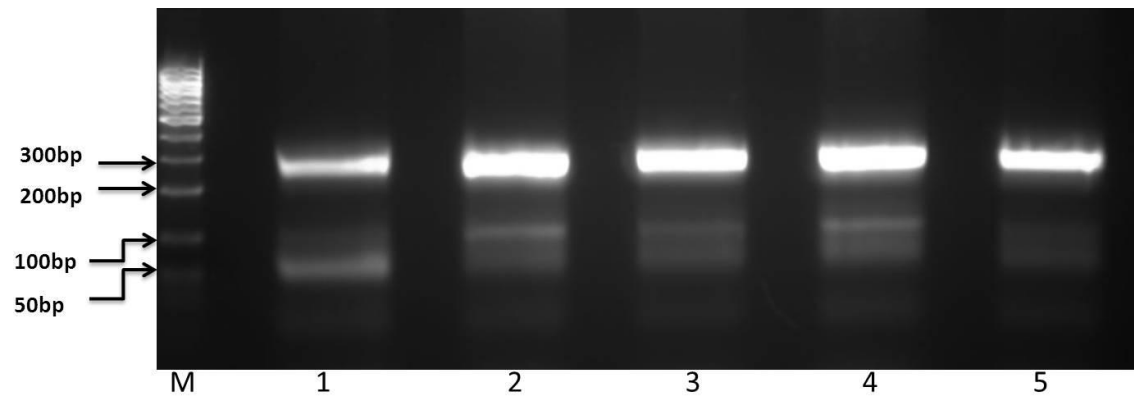


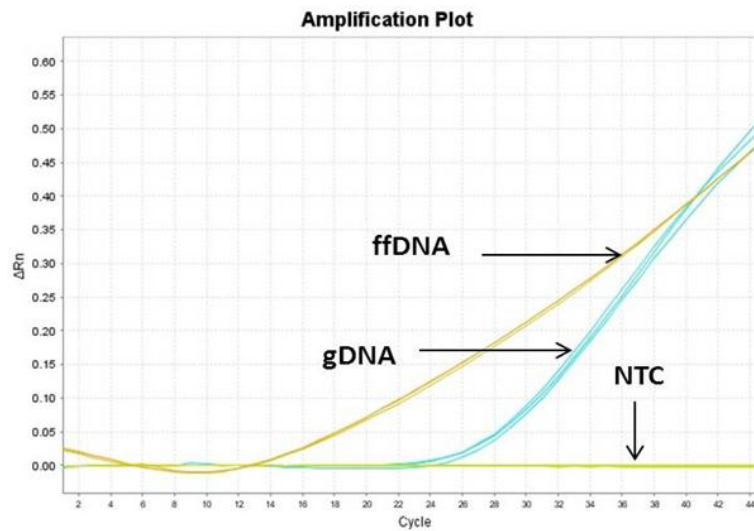
Figure 3: Agarose gel showing fake fetal DNA fragment amplified by PCR with the D21S1890 external forward and reverse primers. M= DNA ladder, lane 1-5 are identical, each containing 110 μ l (100 μ l of PCR product from female gDNA template + 10 μ l of loading buffer).

3.2 RT-PCR to find critical denaturation temperature

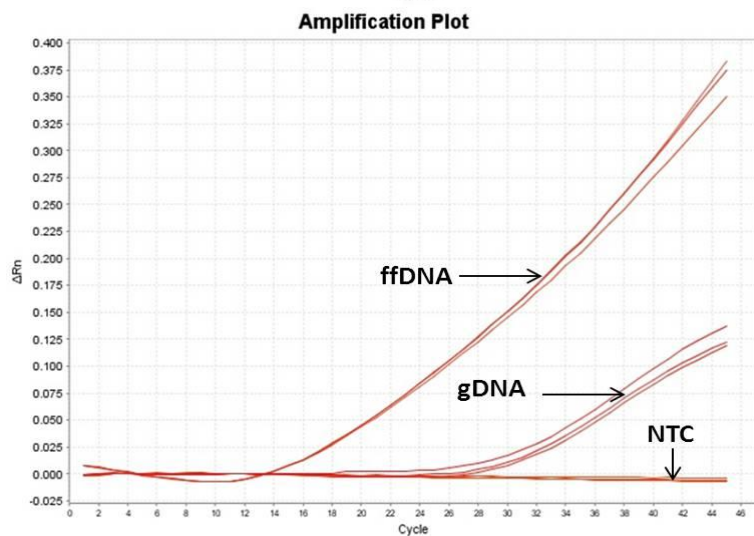
Fake fetal DNA was used to find critical denaturation temperature, but before running fake fetal DNA as a template, different concentrations of primers and probe were used for making a mastermix as shown in Table 2 in an attempt to find best results from these attempts and then running fake fetal DNA as template with chosen conditions to find critical denaturation temperature. Results from these attempts are shown in Appendix B.

COLD-RT PCR was carried out at a denaturation temperature of 78.8 °C and yielded no product from any templates (fake fetal and genomic DNA). This temperature was chosen as previous investigation in Neil Avent's laboratory (unpublished data) had suggested this could be the critical denaturation temperature of this region (using endpoint PCR). To find the critical denaturation temperature the procedure was run multiple times using the same templates with the same reaction conditions. Starting from this point (78.8 °C), a range of different denaturation temperatures were tried to find the critical denaturation temperature (84 °C - 83 °C - 82 °C - 81 °C - 81.8 °C - 81.6 °C - 81.4 °C - 81.2 °C - 80 °C) at which there would be no amplification of female genomic DNA but with the amplification of fake fetal DNA. The first run was done using D21S1890 external forward and reverse primers with fake fetal DNA and female genomic DNA as templates at a denaturation temperature of 95 °C as a positive control. Result from Figure 4 A indicates that both fetal and genomic DNA samples were amplified. Mean Ct value of fake fetal DNA and female genomic DNA was 11 and 26 respectively at a denaturation temperature of 95 °C. Results from other denaturation temperatures, for example 84 °C,

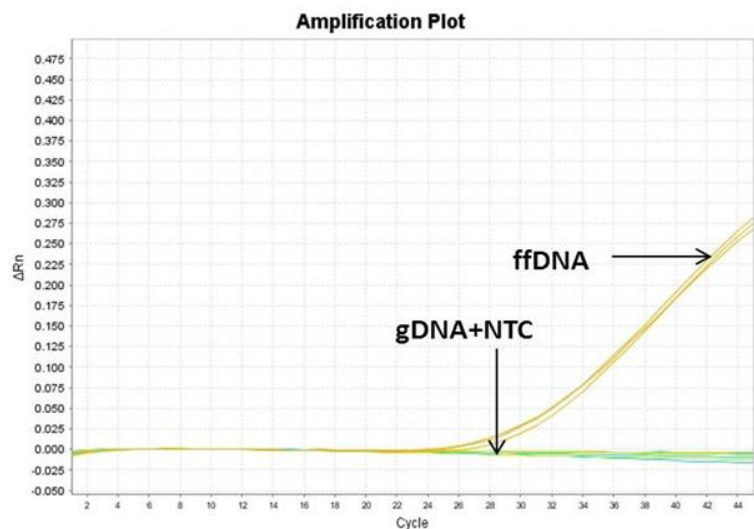
showed no amplification of genomic DNA, however fake fetal DNA showed amplification with very late Ct value of 31.09 as presented in Figure 3 B. The temperature of 80 °C was selected as critical denaturation temperature since the aim was to amplify only fake fetal DNA, indicating that this denaturation temperature is too low for genomic DNA amplification. Mean Ct value for genomic DNA was undetermined at a critical denaturation temperature of 80 °C while mean Ct value for fake fetal DNA was 29. No template control (NTC) samples (in triplicate) did not show any contamination, indicating no amplification of product for all denaturation temperatures (denaturation temperature of 95 °C, 80 °C and 84 °C) Results of other denaturation temperatures that have been tried are shown in Appendix C1 and C2.



A) RT-PCR amplification plot at a Td of 95 °C.



B) RT-PCR amplification plot at a Td of 84 °C.



C) RT-PCR amplification plot at a Td of 80 °C.

Figure 4: RT-PCR amplification plots to determine critical denaturation temperature. **A)** At a Td of 95 °C, the mean Ct value was 11 and 26 for fake fetal DNA (ffDNA) (0.02ng/μl) and gDNA (20ng/μl) respectively. **B)** At a Td of 84 °C, the mean Ct value was 31.09 for ffDNA with undetermined Ct value for gDNA. **C)** At a critical Td of 80 °C the mean Ct value was 29.70 for ffDNA with undetermined Ct value for gDNA. No template control (NTC) traces show no amplification indicating that there were no contamination at all temperatures.

3.3 Fragment analysis to find fake maternal DNA

End point PCR using 5'FAM labelled external forward and non-labelled external reverse primers produced labelled DNA fragment (Method 2.3.2) from twenty four different genomic DNA samples (derived from buffy coat). These samples were compared with female genomic DNA which fake fetal DNA was produced from, in order to find fake maternal DNA for spiking experiment. The CA repeat in chromosome 21 marker region varies within the population. For the reason that every individual inherits one allele from their mother and one from their father, we expected to see two major peaks from each of these samples. By comparing the peaks from the different samples with the female genomic DNA we wanted one of these major peaks to match and the other peak to not match. Using this principle, we have chosen the sample to act as fake maternal DNA for the spiking experiment.

A portion of the PCR products were initially run for forty five minutes at ninety volts across an agarose gel. This was to verify that product had been amplified and that it was of the correct size. The gel photo showed that all templates had given a good strong band at approximately 229 bp as expected; these results are shown in Appendix D. Only a small volume 1-2 µl of remaining PCR was then assessed by Dr. Michele Kiernan using an ABI Genetic analyser 3130, and the information analysed using Peak ScannerTM Software version 1.0 to produce electropherograms. All fragments identified stutter peaks. Some of these results are presented in Figure 5. These results led us to choose 6534H as a fake maternal DNA. However the stutter peaks were clear from these results which could be due to CA repeat as it is dinucleotide repeat polymorphism. Other results of fragment analysis are shown in Appendix E.

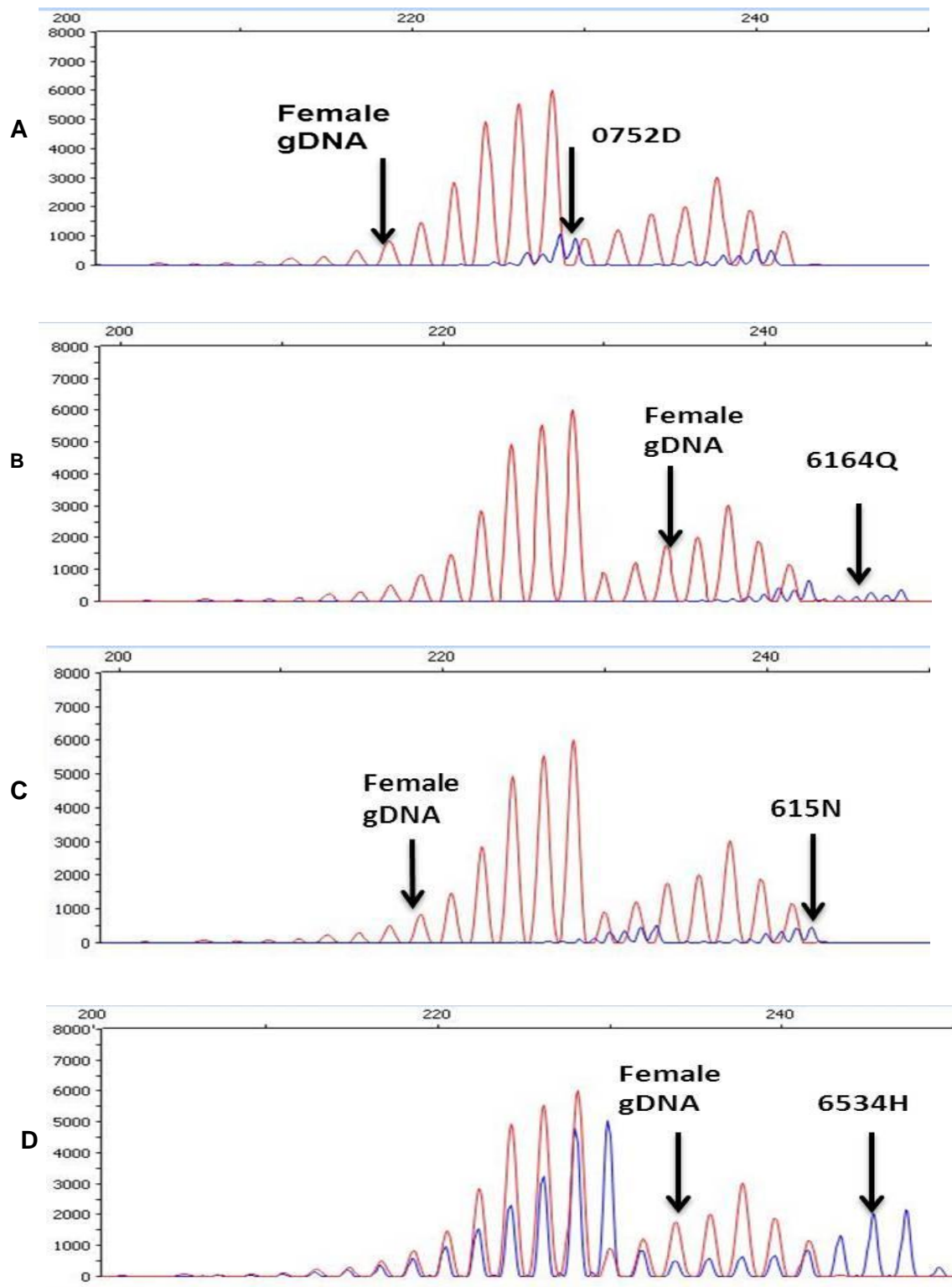


Figure 5: Electropherograms (derived from Peak Scanner TM software) for selecting fake maternal DNA sample. Y axis represents signal intensity peak height and X axis represents size base pairs. D21S1890 FAM labelled external forward and reverse primers were used at Td of 95 °C, the templates were different gDNA samples derived from buffy coat. **A)** Female gDNA is shown in red, and 0752D is shown in blue. Figures **B**, **C** and **D** are with the same female gDNA (red colour) compared with 6164Q, 615N and 6534H (all in blue) gDNA respectively. Sample 6534H was regarded as fake maternal DNA as it is clear from figure **(D)** that some peaks match female gDNA peaks and others are not matching.

3.4 Performing PCR with internal primers for fake fetal and maternal DNA

End point PCR was performed for both fake fetal and fake maternal DNA, using D21S1890 FAM labelled internal forward and non-labelled internal reverse primers. AmpliTaq Gold enzyme was used as the first attempt for running these samples with internal primers at denaturation temperatures of 95 °C and 80 °C (expected product size of 101 bp). An aliquot of PCR amplicons was run on an agarose gel to check for the product size, the remaining PCR products were run on genetic analyser for fragment analysis.

At a denaturation temperature of 95 °C with using AmpliTaq Gold enzyme, Figure 6 A shows an expected size product (101 bp) for both fake fetal and fake maternal DNA. However, fake maternal DNA shows faint band and a smaller band other than main band (Figure 6 A, lane 2) comparing to fake fetal DNA product at the same denaturation temperature, there is possible contamination as the negative control at both temperatures indicated contamination. While at a critical denaturation temperature of 80 °C, the result shows no amplification for the templates. The gel photo only shows primer dimer as seen in Figure 6 A lane 4 and lane 5. Results of running these samples on genetic analyser are presented in Appendix F.

Moreover we have used AmpliTaq Gold enzyme with an extra step of heating mastermix, first at 95 °C for fifteen minutes in a separate PCR tube prior to adding templates. The result from this attempt was also unsatisfactory (Appendix G) as the gel photo showed no amplification of fake fetal and fake maternal DNA at a critical denaturation temperature of 80 °C.

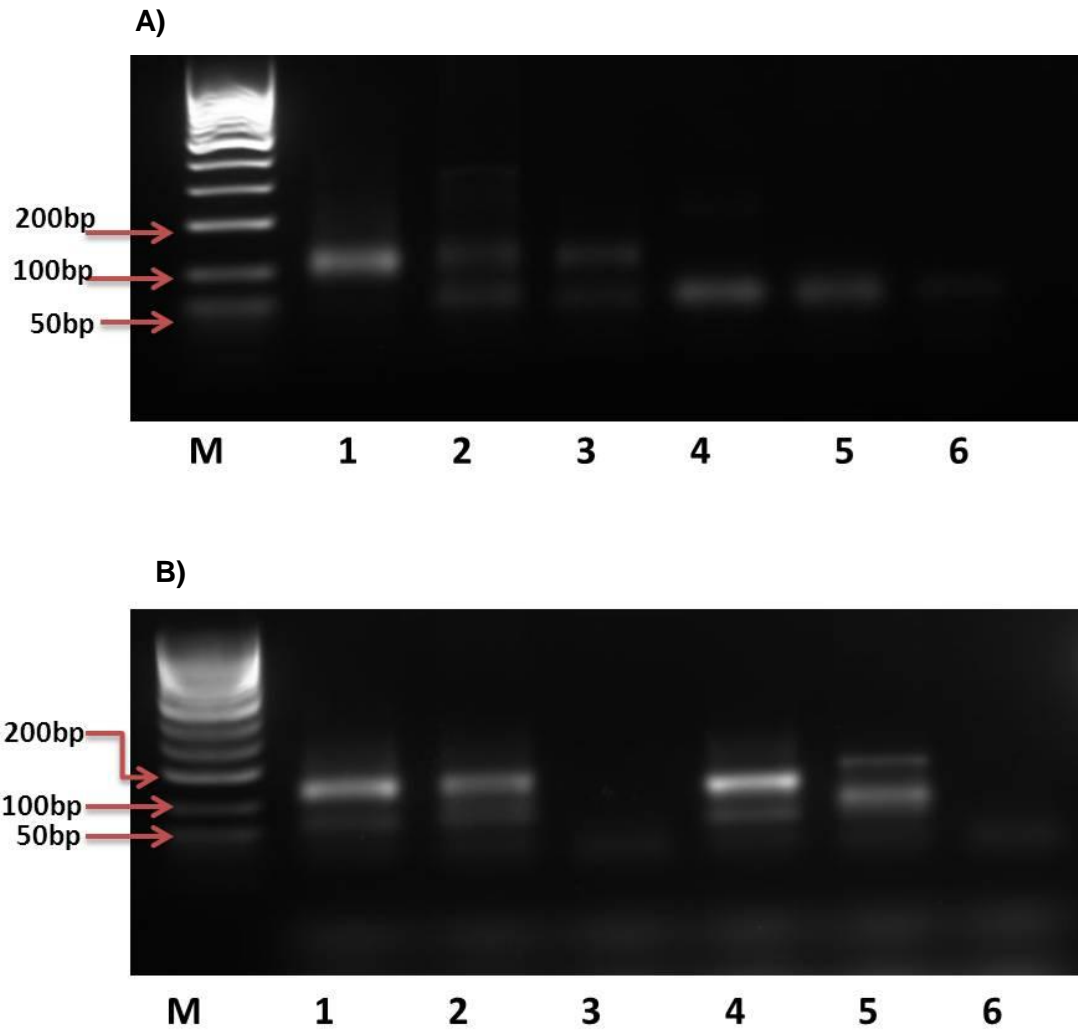


Figure 6 : Agarose gel electrophoresis for PCR with D21S1890 FAM labelled internal forward and reverse primers using both AmpliTaq Gold and TaqMan mastermix.

A) Using AmpliTaq Gold enzyme: lane M = DNA ladder, Lane 1 = fake fetal DNA at a Td of 95 °C, Lane 2 = fake maternal DNA (6534H) at a Td of 95 °C, Lane 3 = NTC. Lane 4, 5 and 6 same as lane 1, 2, and 3 but at a critical Td of 80 °C.

B) Using TaqMan universal PCR mastermix: lane M = DNA ladder, lane 1 = fake fetal DNA at a Td of 95 °C, lane 2 = fake maternal DNA (6534H) at a Td of 95 °C and lane 3 = NTC. Lane 4, 5 and 6 same as lane 1, 2, and 3 but at critical Td of 80 °C.

TaqMaster PCR Enhancer was also tried which is a buffer additive that improves thermostability (enzyme half-life) and processivity of Taq DNA polymerase by stabilizing the enzyme during PCR. It does not change the optimal primer annealing temperature or the melting behaviour of template DNA. PCR Enhancer was used with both AmpliTaq Gold enzyme and also with TaqMan mastermix. However, the agarose gel results indicated no difference between running these samples with or without PCR Enhancer as shown in Appendix H. Furthermore, the remaining portion of these PCR amplicons were run on genetic analyser for fragment analysis, to see if there is any possibility for stutter peaks elimination. However the electropherograms showed stutter peaks from running these samples with PCR Enhancer (Appendix I).

Lastly TaqMan universal PCR mastermix was used as we knew that it definitely worked at both denaturation temperatures, as shown by the results of RT-PCR. Figure 6 B shows the results with using TaqMan indicating heteroduplex formation at both temperatures. At a denaturation temperature of 95 °C the result shows product with expected size of 101 bp for both fake fetal and fake maternal DNA (Lane 1 and Lane 2). While at critical denaturation temperature of 80 °C the result shows clear product for fake fetal DNA and unexpected product for fake maternal DNA with different product size (larger product size) (Figure 6 B, lane 4 and 5). NTC lanes show no contamination at both denaturation temperature (Figure 6 B, lane 3 and 6).

These final results (using TaqMan mastermix and with D21S1890 internal primers) were run on genetic analyser and compared with same samples but with D21S1890 external primers at a denaturation temperature of 95 °C. Figure 7 indicates that the peak patterns look the same, but the shift in size of the product due to the different primers used for the PCR, and also there are enough differences between fake maternal and fake fetal DNA.

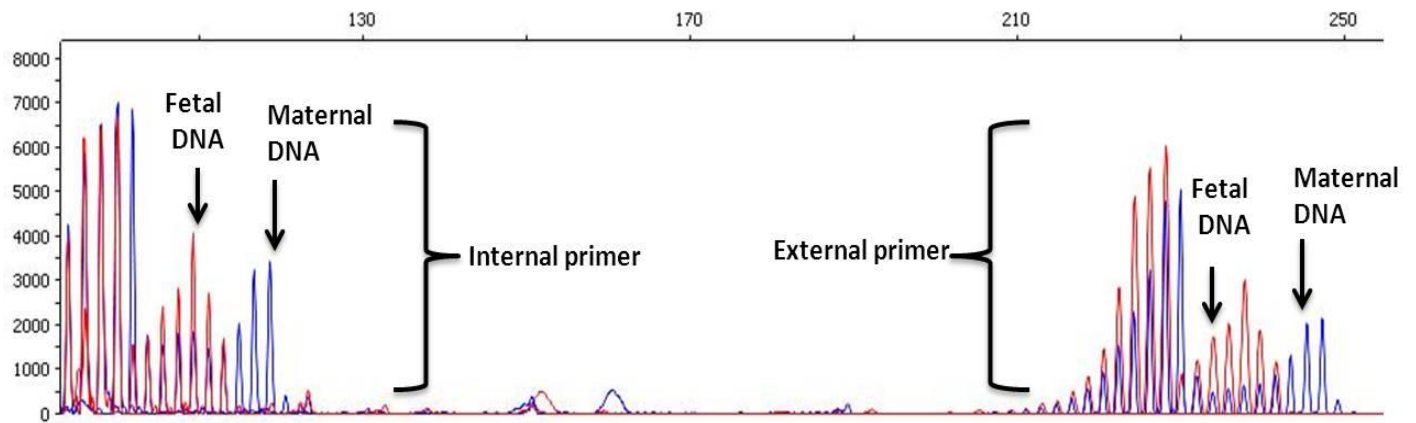


Figure 7 : Electropherogram (derived by Peak Scanner TM software) for both fake fetal and fake maternal DNA. Using internal and external D21S1890 primers, Y axis represents signal intensity peak height and X axis represents size base pairs. Maternal DNA (blue colour) and fetal DNA (red colour)

3.5 Spike experiment

3.5.1 RT-PCR results

The aim of the spiking experiment was to test if the fake fetal fragment would denature without denaturation of fake maternal DNA at critical denaturation temperature of 80 °C. After determining critical denaturation temperature by RT-PCR, the spike experiment was performed, with fake fetal and fake maternal DNA being used as templates. As mentioned before (Method 2.5) four plates were prepared; two plates were run on StepOnePlus and the other two plates on the Veriti. On the StepOnePlus, one plate worked as positive control that all samples (small fake fetal fragment and large fake maternal DNA) would denature at 95 °C, while at 80 °C small fetal fragment would denature but not fake maternal DNA. For StepOnePlus we have used non-labelled internal D21S1890 forward and reverse primers and labelled D21S1890 probe. While for Veriti/end point PCR we used D21s1890 FAM labelled internal forward and non-labelled internal reverse primer.

Figure 8 A and B shows standard curves (STD) at denaturation temperatures of 95 °C and 80 °C, fake maternal DNA was used as STD at 95°C, while fake fetal DNA was used as STD at 80 °C. These results shows mean Ct value of 29 for STD at a denaturation temperature of 95 °C, and mean Ct of 28 for STD at a critical denaturation temperature of 80 °C.

At both denaturation temperatures NTC samples show no contamination as seen in Figure 8 C and D. The spike sample with composition of 90% of fake maternal DNA and 10% of fake fetal DNA shows amplification at both denaturation temperatures with mean Ct value of 6 at a denaturation temperature of 95°C and 26 at a critical denaturation temperature of 80°C

(Figure 8 C and D). Ct value of spike sample corresponds with fake fetal DNA at 80°C which was 25 as shown in Figure 9 C. Fake maternal DNA showed amplification at a denaturation temperature of 95 °C with mean Ct value of 26 (Figure 9 A), which is the same as Ct value for genomic DNA as presented in Figure 4 A, while Figure 9 B shows no amplification of fake maternal DNA at a critical denaturation temperature of 80 °C as expected. Results of spike sample with composition of 95% fake maternal DNA and 5% fake fetal DNA are shown in (Appendix K1 and K2).

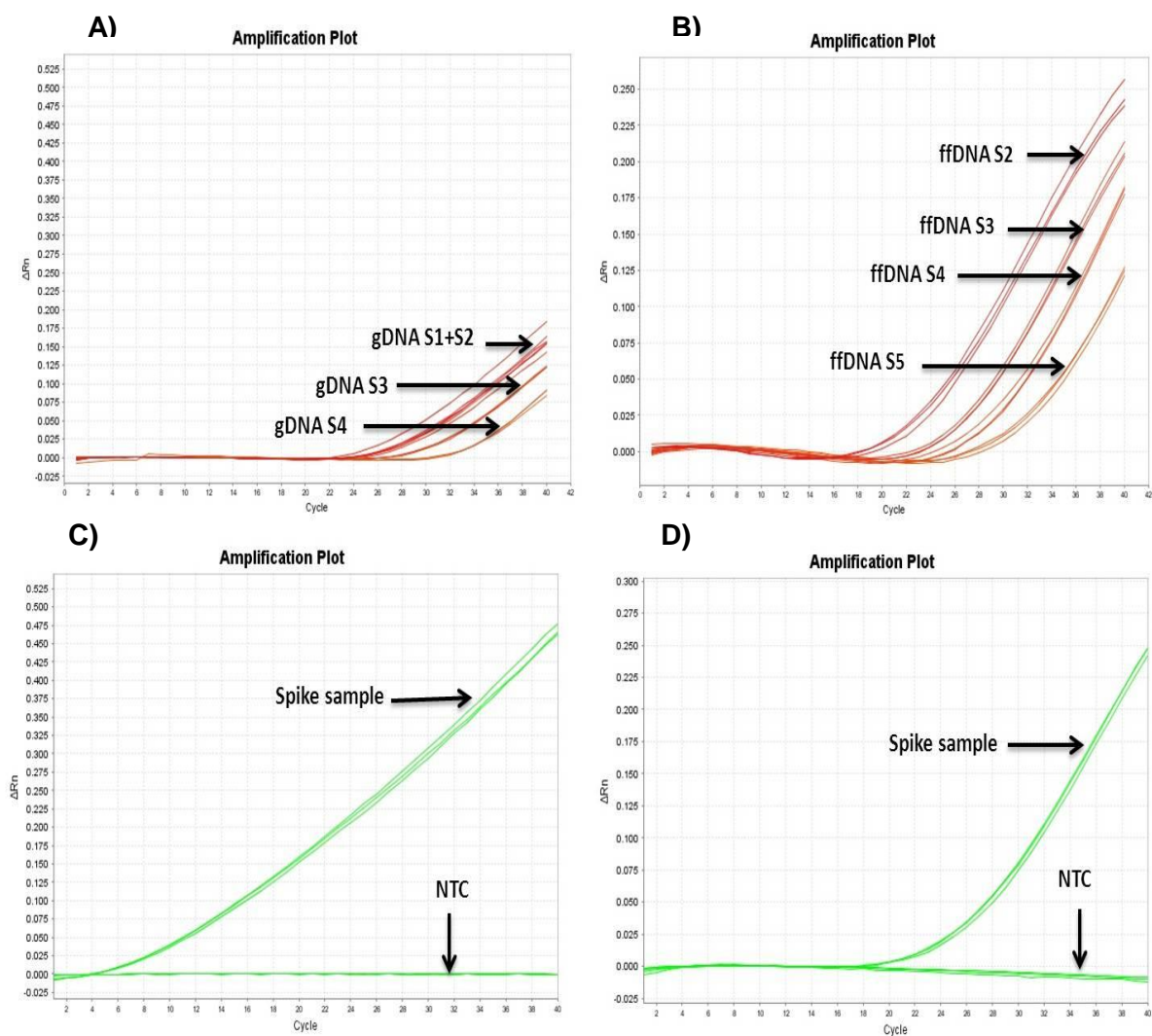


Figure 8: RT-PCR amplification plots of spike experiment showing standard curve (STD) and spike sample at Td of 95 °C and 80 °C. **A)** Shows the STD of fake maternal DNA at a Td of 95 °C using different starting template dilutions, S1 (20ng/ μ l), S2 (2ng/ μ l), S3 (0.2ng/ μ l) and S4 (0.02ng/ μ l). **B)** Shows standard curves of fake fetal DNA at critical Td of 80 °C using starting template dilutions S2 (2ng/ μ l), S3 (0.2ng/ μ l), S4 (0.02ng/ μ l) and S5 (0.002ng/ μ l). Figure **C)** and **D)** shows spike sample (90% fake maternal DNA: 10% fake fetal DNA) at Td of 95 °C and 80 °C respectively. NTC shows no contamination at both denaturation temperatures.

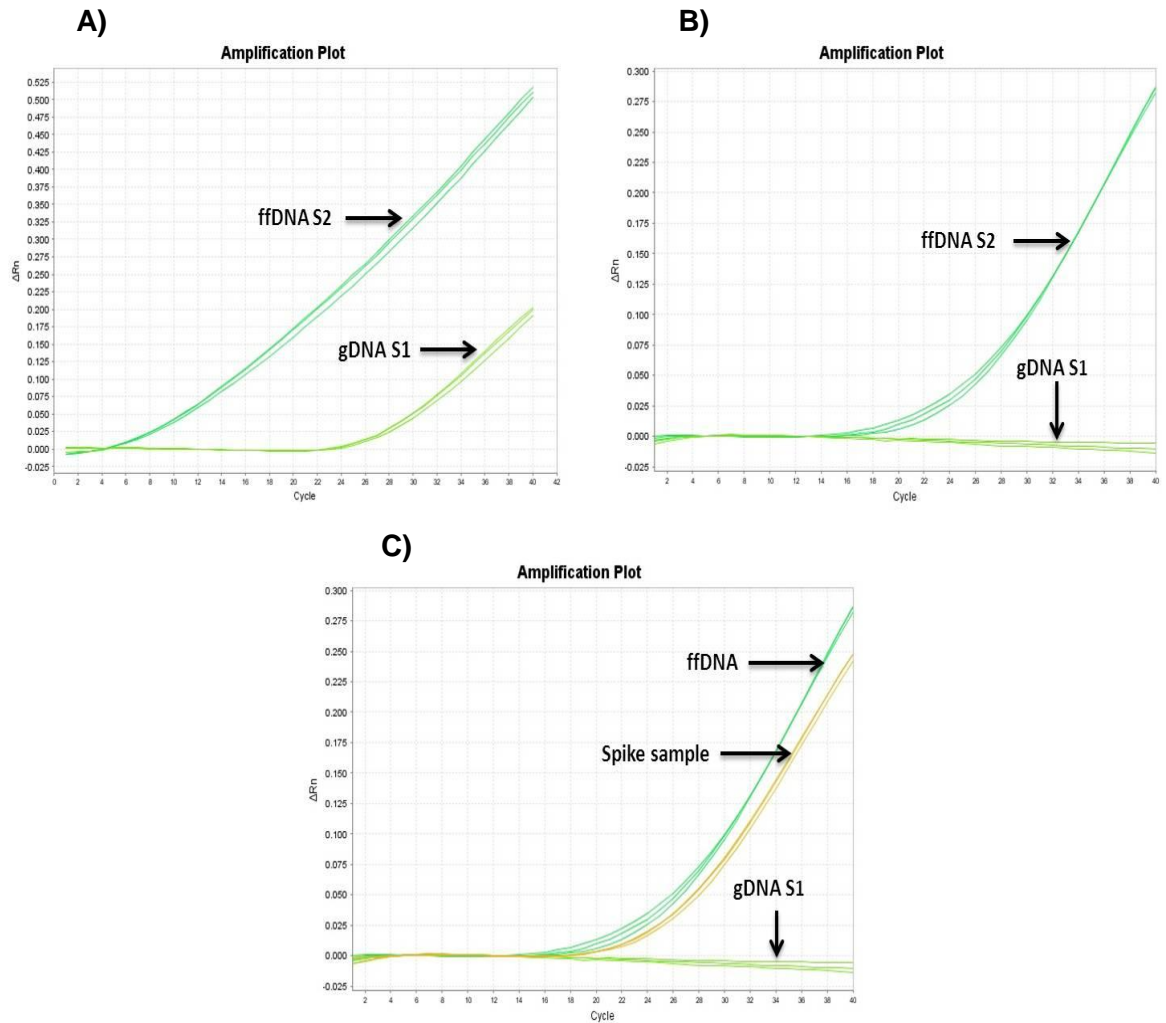


Figure 9: RT-PCR amplification plots of spike experiment showing both fake fetal and fake maternal DNA at Td of 95 °C and 80 °C, using TaqMan probe and D21S1890 internal forward and reverse primers. **A)** At a Td of 95 °C the amplification plot shows amplification of both templates [fake fetal DNA (0.4 ng/ μ l) and fake maternal DNA (4 ng/ μ l)]. **B)** At critical Td 80 °C fake fetal DNA shows amplification while fake maternal DNA is not amplified. **C)** Shows that both spike sample and fake fetal DNA are amplifying at critical Td of 80 °C with mean Ct of 26 and 25 respectively (without amplification of fake maternal DNA (Ct value undetermined)).

Samples (fake fetal DNA, fake maternal DNA and spike sample) were run out on agarose gels to check the size of the products. At a denaturation temperature of 95 °C Figure 10 A shows amplification of all templates, however the product from fake maternal DNA presents as a faint band (lane 7 and lane 8) compared to the product of other templates at the same temperature.

While at a critical denaturation temperature of 80 °C the result shows unexpected product for fake maternal DNA, furthermore the result shows a different product size (larger product size) as shown in lane 7 and lane 8 figure 10 B. These results were consistent with the results from Figure 6 A. However running these same samples on an agarose gel showed product for fake maternal DNA at a critical denaturation temperature of 80 °C, which was unexpected, as RT-PCR results showed no amplification of fake maternal DNA at a critical denaturation temperature of 80 °C (Figure 9 B). The bands of fake fetal and spiked sample looked similar to each other on the gel which corresponds with RT-PCR results (both spiked sample and fetal DNA had mean Ct of 26 and 25 respectively).

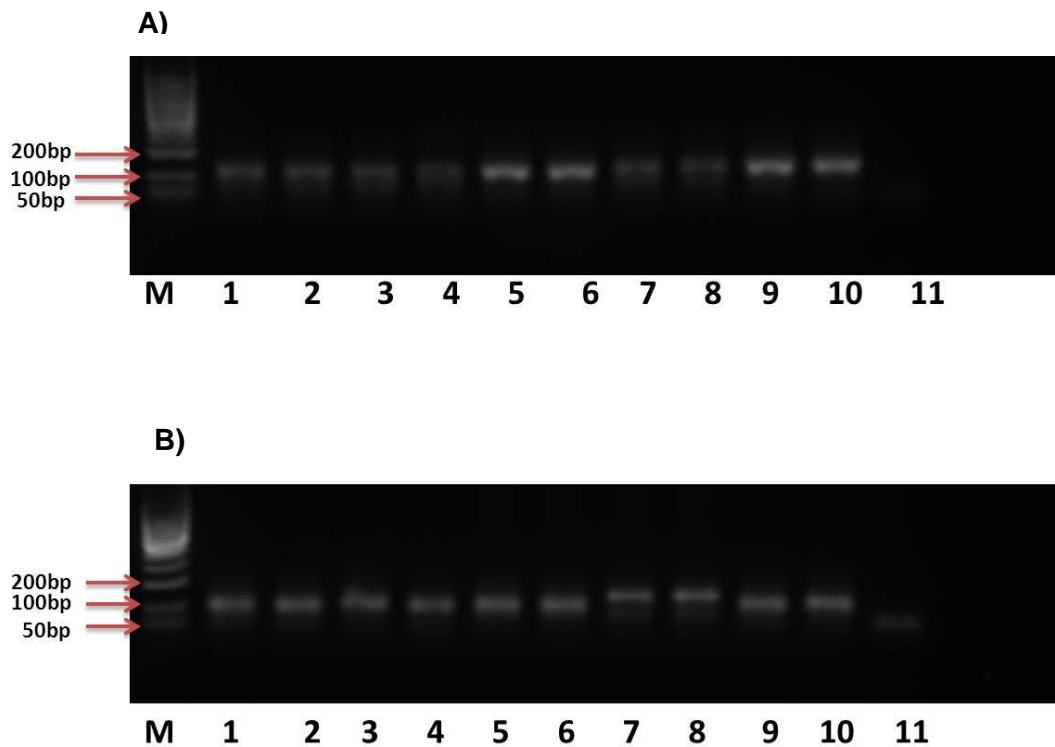


Figure 10: Agarose gel of spiking experiment at Td of 95 °C and 80 °C. D21S1890 FAM labelled internal forward and reverse primers were used.

A) Shows the results at a Td of 95°C:

M = DNA ladder, lane 1, 2, 3 and 4 are standard curve of fake maternal DNA (6534H) at a final concentration of 4 ng/μl, 0.4 ng/μl, 0.04 ng/μl and 0.004 ng/μl respectively.

Lane 5= spike sample with composition of (90% maternal DNA + 10% fetal DNA)

Lane 6 = spike sample with composition of (95% maternal DNA and 5% fetal DNA)

Lane 7= fake maternal DNA (90%)

Lane 8 = fake maternal DNA (95%)

Lane 9 = fake fetal DNA (10%)

Lane 10 = fake fetal DNA (5%)

Lane 11 = NTC

B) Shows the results of 80 °C critical Td, lane 1, 2, 3 and 4 are standard curves of fake fetal DNA at a final concentrations of 0.4 ng/μl, 0.04 ng/μl, 0.004 ng/μl and 0.0004 ng/μl respectively, the samples were loaded in lanes 5-11 in the same order as in figure 9 A.

The same products were run out on an acrylamide gel again to double check the product size difference. Figure 11 A shows the product from all samples (Spike sample, fake fetal and fake maternal DNA) at a denaturation temperature of 95 °C, however the fake maternal DNA bands looks extremely faint compared to that at 80 °C (Figure 11 B) which was completely unexpected. At a critical denaturation temperature of 80 °C, the product generated from both spiked sample and fake fetal DNA are smaller than expected size, they were both different from fake maternal DNA which presented in clear band and higher product size (Figure 11 B lane 3 and lane 4).

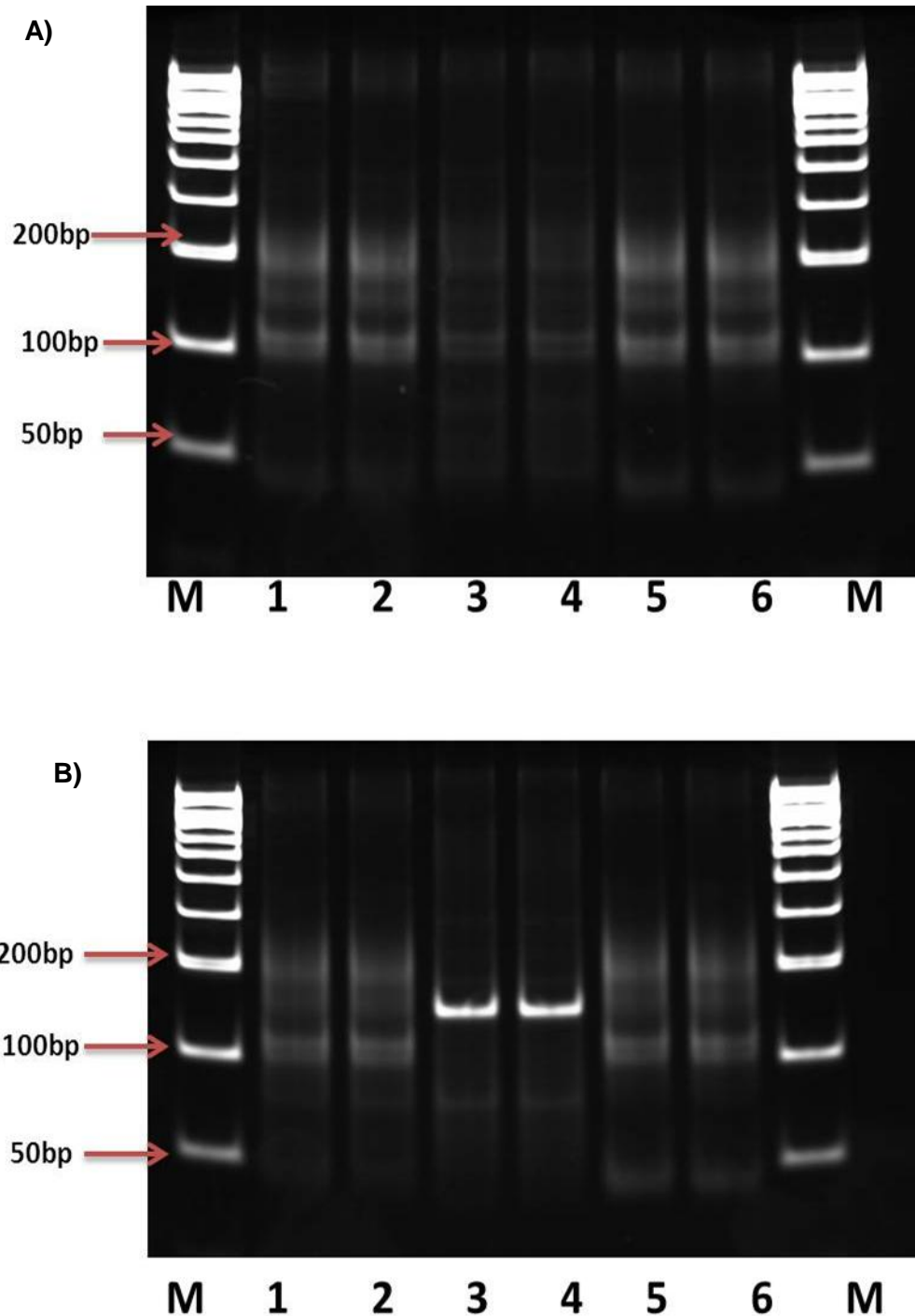


Figure 11: Non denaturing polyacrylamide gel electrophoresis result of spiking experiment at Td of 95 °C and 80 °C Td, Stained with Gel Red. **A)** Shows the results at a Td of 95 °C run: M =DNA ladder, lane 1 = spike sample with composition of 90% maternal DNA + 10% fetal DNA, lane 2 = spike sample with composition of 95% maternal DNA and 5% fetal DNA, lane 3= fake maternal DNA (90%), lane 4 = fake maternal DNA (95%), lane 5 = fake fetal DNA (10%), lane 6 = fake fetal DNA (5%). **B)** Shows the results at a critical Td of 80 °C, the order of loading for the samples were The same as in figure 10 A.

3.5.2 Fragment analysis result

Figure 12 shows the fragment analysis result for the spike experiment at denaturation temperatures of 95 °C and 80 °C. At a denaturation temperature of 95 °C the peaks were coming from all samples (fake fetal DNA, fake maternal DNA and spike sample), which confirms the result from RT-PCR (which showed amplification from all templates at a denaturation temperature of 95 °C). At critical denaturation temperature 80 °C fragment analysis result showed absence of fake maternal DNA peaks. It is not complete absence but nevertheless it is not the same as peaks of fake maternal DNA at a denaturation temperature of 95 °C.

The spike sample (90% maternal DNA and 10% fetal DNA) matches fake fetal DNA in both electropherograms. We would expect this match at critical denaturation temperature 80 °C but at 95 °C the spike sample was expected to also correspond to the maternal peaks but it does not as shown in figure 12 A and B. Also these results indicate that the product of spike sample is predominantly from fake fetal DNA sample and not from fake maternal DNA. Again this was supported by the same result of RT-PCR result which showed close Ct value between fake fetal and spike sample at a critical denaturation temperature of 80°C (mean Ct value of 25 and 26 respectively) with no amplification of fake maternal DNA (Ct value undetermined).

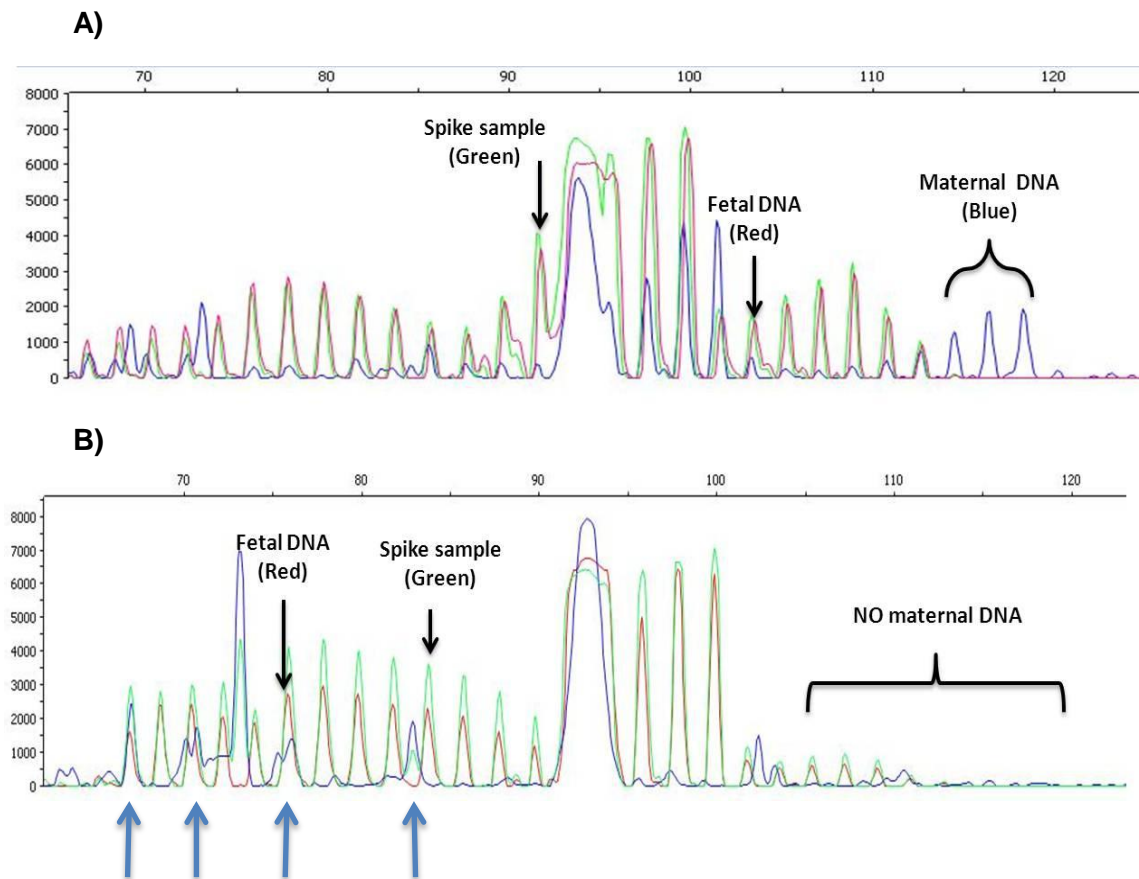


Figure 12: Electropherogram derived by (Peak Scanner TM software) for the spike experiment at Td of 95 °C and 80 °C. Y axis represents signal intensity peak height and X axis represents size in base pairs. D21S1890 FAM labelled internal forward and reverse primers were used. **A)** Shows result at a Td of 95 °C, spike sample (green), fake maternal DNA sample (blue) and fake fetal DNA (red). **B)** Shows the results at critical Td 80 °C, spike sample (green), fake maternal DNA sample (blue) and fake fetal DNA (red). The area of graph B where there is absence of maternal peaks is labelled clearly showing that maternal fragments have failed to be amplified in this region by COLD-PCR. Blue arrows point out few maternal peaks at Td of 80°C

3.5.3 SYBR Green assay

3.5.3.1 RT-PCR result

RT-PCR using SYBR Green mastermix was performed, using D21S1890 internal forward and reverse primers and the templates were spike sample (90% fake maternal DNA + 10% fake fetal DNA), fake maternal DNA alone (90 %) and fake fetal DNA alone (10 %); again at two different denaturation temperatures (95°C and 80°C).

The RT-PCR results indicate that at a critical denaturation temperature of 95 °C fake maternal DNA, fake fetal DNA and spike sample are all amplified. The mean Ct value for the fake maternal DNA, fake fetal DNA and spike samples were 3, 24 and 4 respectively as shown in Figures 13 A and C. At critical denaturation temperature of 80 °C fake maternal DNA showed amplification with Ct value of 32 (Figures 13 B and E). This result was unexpected as the results of RT-PCR with TaqMan probe showed no amplification of fake maternal DNA (Figure 9 B), and also the results of RT-PCR for identifying critical denaturation temperature showed no amplification of genomic DNA sample at 80°C as presented in Figure 4 B. Both fake fetal DNA and spike sample showed amplification at 80 °C critical denaturation temperature with mean Ct value of 27 for fake fetal DNA and 15 for spike sample. The negative water control at denaturation temperature of 95 °C and 80 °C indicated contamination as shown in Figures 13 C and D).

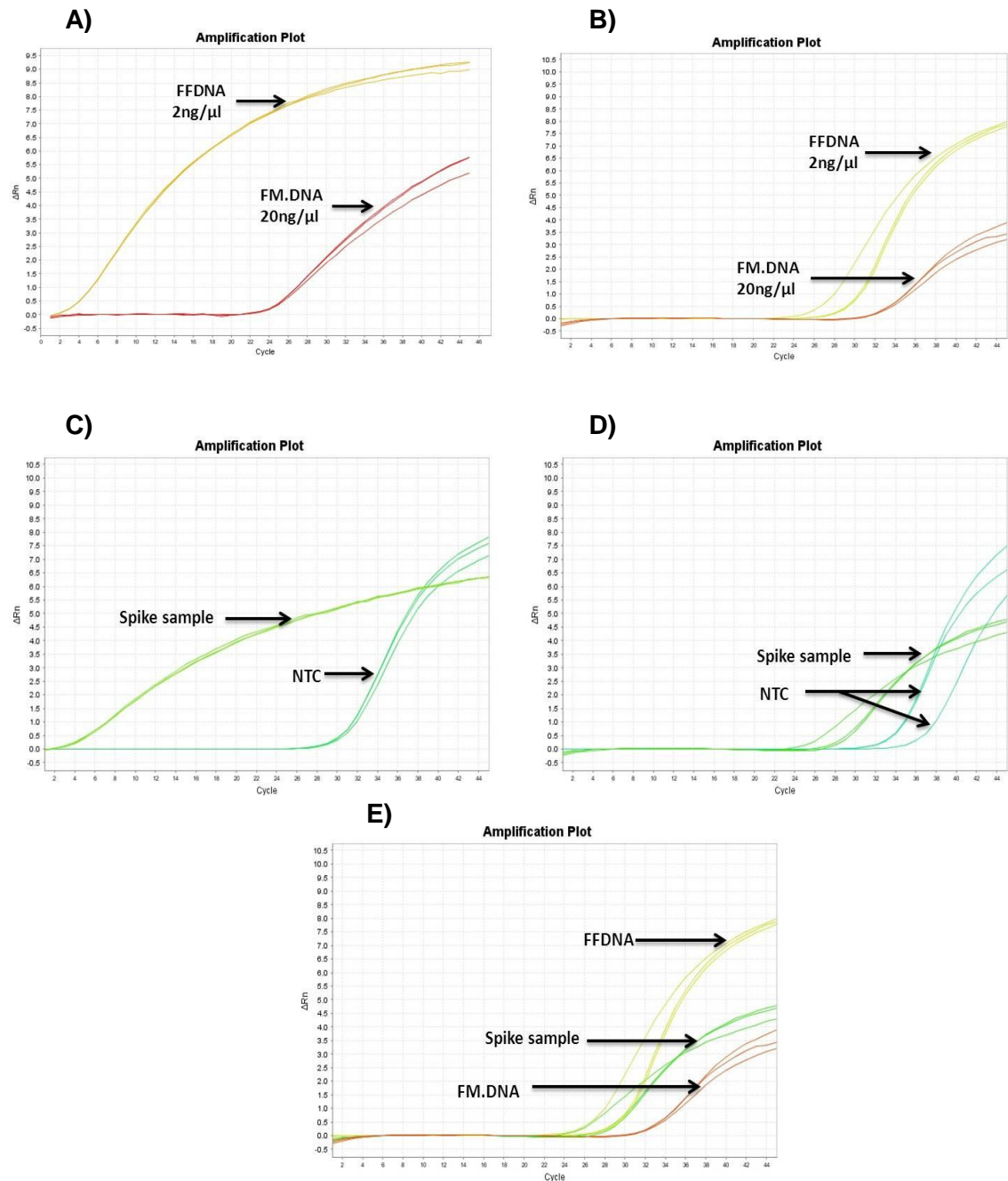


Figure 13: RT-PCR amplification plot for the spike experiment using SYBR Green at Td of 95 °C and 80 °C. **A)** RT-PCR amplification plot at Td of 95 °C showing amplification of both fake fetal and fake maternal DNA with mean Ct value of 3 and 24 respectively. **B)** RT-PCR amplification plot at critical Td of 80 °C, showing amplification of fake fetal DNA with mean Ct value of 27 and fake maternal DNA showed amplification with late mean Ct value of 32. **C)** RT-PCR amplification plot at 95 °C showing spike sample (90% fake maternal DNA + 10% fake fetal DNA) with mean Ct value of 4 and negative water control. **D)** RT-PCR amplification plot at 80 °C showing spike sample (90% fake maternal DNA + 10% fake fetal DNA) with mean Ct value of 15 and negative water control. **E)** RT-PCR amplification plot at 80 °C showing spike sample, fake maternal and fetal DNA.

3.5.3.2 Melt curve analysis

The use of SYBR Green allows melt curve analysis to be performed. Following RT-PCR, the amplicon amplified should have been exactly the same for both fake maternal DNA and fake fetal DNA and therefore each denaturation temperature should have indicated the same melting temperature (T_m) with melt curve analysis. However the results showed that the T_m of the amplicon produced at critical denaturation temperature of 80°C was lower than T_m of amplicon produced from same template at 95°C (Figure 14 A, B and C); this difference was 4.15°C in fake maternal and 3.24°C for the fake fetal DNA, whereas spike sample showed same T_m at both runs. The fake maternal DNA, fetal DNA and spike samples have obvious shoulder peaks at critical denaturation temperature of 80 °C, indicating the amplification of products with differing sequences possibly as a result of heteroduplex formation or contamination (Figures 14 D, E and F). We expected to see the same T_m for both spike sample and fake fetal DNA at the critical denaturation temperature of 80 °C; however the result showed different T_m values, indicating that the spike sample may not solely derive from fake fetal DNA. These results were consistent with RT-PCR results as presented in Figures 13 B and C, in which the replicates of the samples (fake maternal DNA, fake fetal DNA and spike sample) were not in agreement.

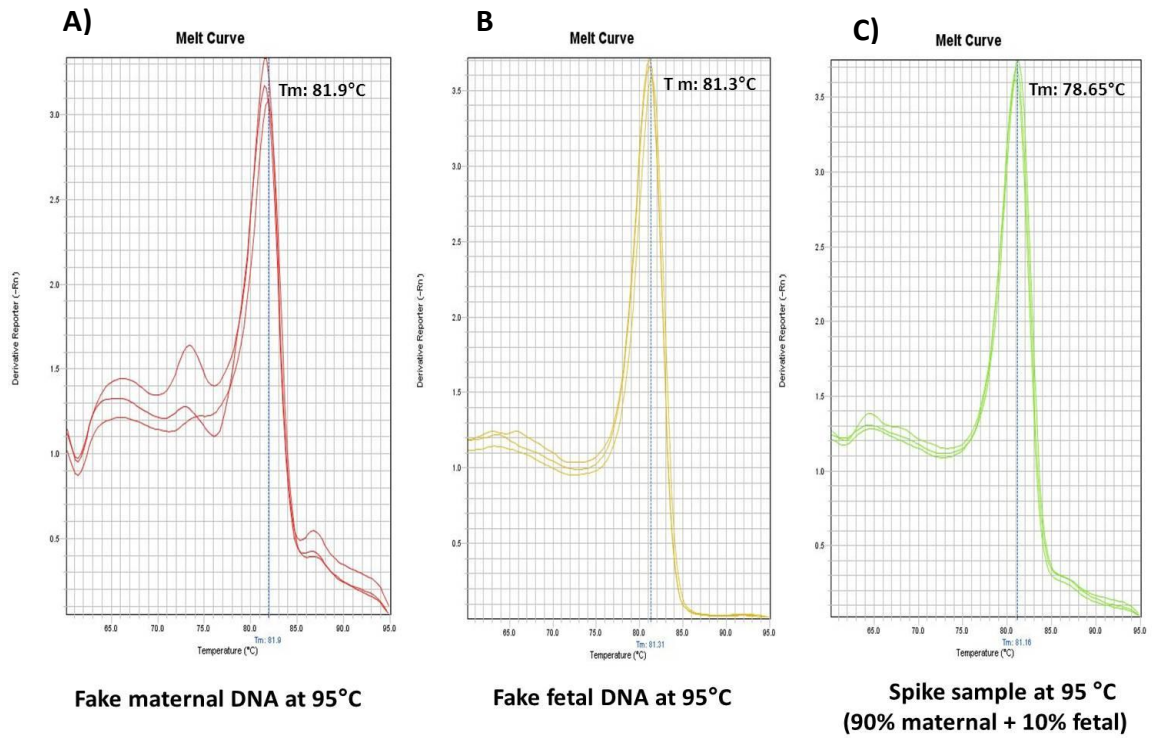
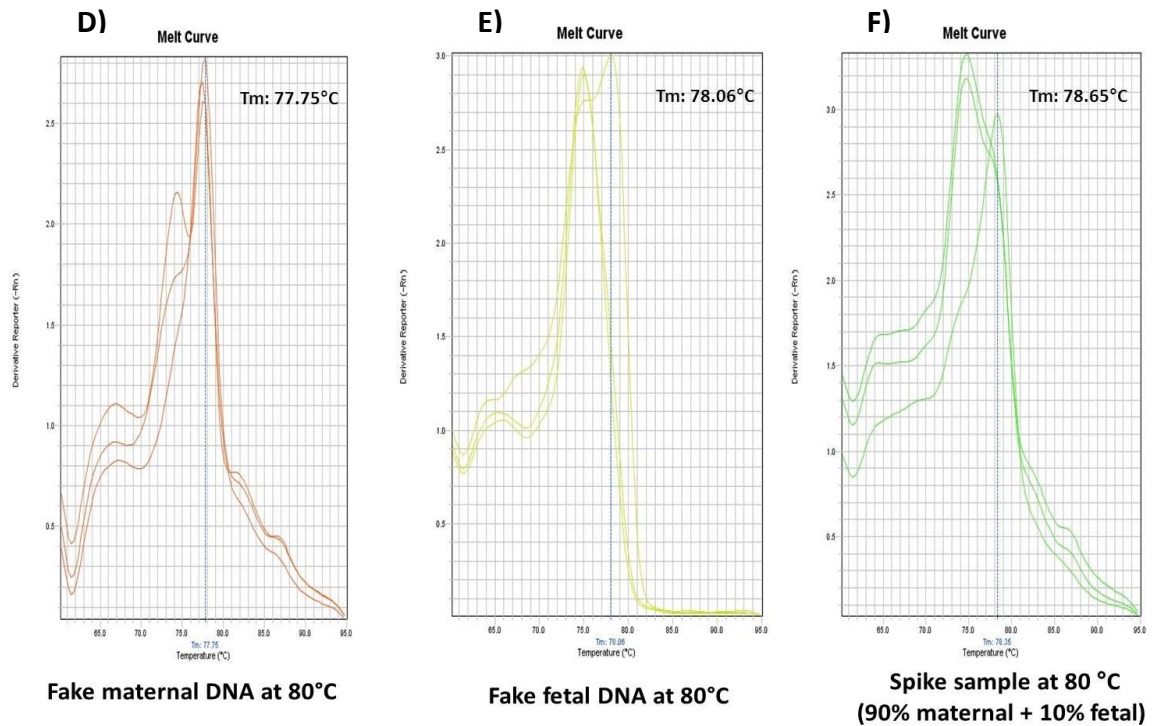


Figure 14: Melt curves of RT-PCR products of spike experiment at Td of 95 °C and 80 °C, showing fake fetal DNA, fake maternal DNA and spike samples. From left to right graphs show the melt curves of fake maternal, fetal and spike templates products. The top row show the curves from the products at Td of 95 °C (A, B and C) and the bottom row show the curves of products at critical Td 80 °C critical denaturation temperature (D, E and F).



3.6 Cloning experiment

The purpose of the cloning experiment was to clone the PCR product at denaturation temperatures of 95 °C and 80 °C for both fake fetal DNA (produced from female genomic DNA) and fake maternal DNA (6534H from buffy coat) and then sequence them to see the representation of CA repeat. TOPO TA Cloning[®] Kit for Sequencing was used for this purpose.

Fake fetal and fake maternal DNA were used as templates to perform a PCR with D21S1890 internal forward and reverse primers, at denaturation temperatures of 95 °C and 80 °C. This was performed to generate insert (expected size 101 bp) to be used for the cloning experiment. At the same time another PCR was performed with D21S1890 external forward and reverse primers, for fake fetal DNA and female genomic DNA at a denaturation temperature of 95 °C, this was to generate insert (expected size 229 bp) for the cloning experiment. Figure 15 shows agarose gel electrophoresis result for these PCR products (with denaturation temperatures 90°C and 80°C). These results indicate a clear product amplification of around 229 bp (lane 1= fake fetal DNA and lane 2= fake maternal DNA), both at a denaturation temperature of 95 °C. However running same templates with D21S1890 internal forward and reverse primers showed smaller products as well as the expected amplified fragment (101 bp) (Figure 15 lane 3 and 4 at 95 °C; lane 5 and 6 at 80 °C), therefore gel purification was done. Instead of having two samples at each temperature the total numbers of samples was four samples at each temperature. Once DNA had been purified it was quantified using the Nano Vue Plus (GE Healthcare) spectrophotometer. The concentrations were calculated from absorbance reading and are presented in Table 6.

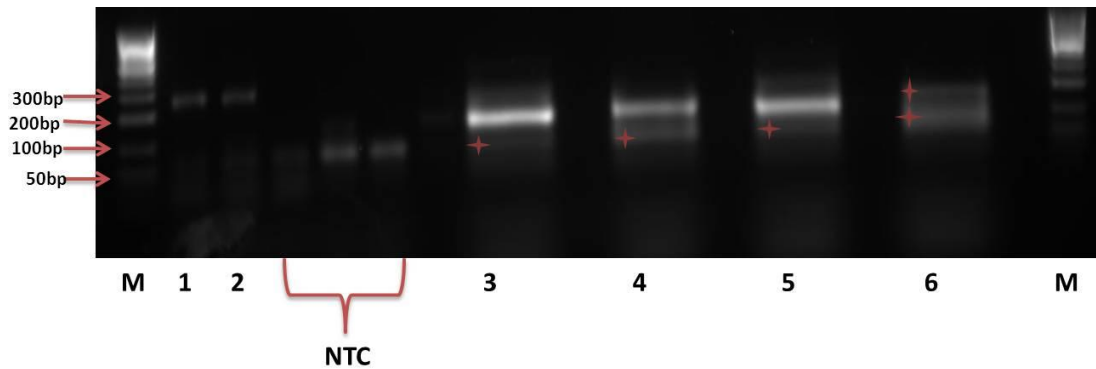


Figure 15: Agarose gel of PCR for the cloning experiment at Td of 95 °C and 80 °C M=DNA ladder, lane 1= fake fetal DNA, lane 2= fake maternal DNA, both at Td of 95 °C using D21S1890 external forward and reverse primers. Approximately 229 bp PCR amplicon was generated and used as insert for the cloning experiment. Lane 3= fake fetal DNA, lane 4= fake maternal DNA both at a Td of 95 °C but with D21S1890 internal forward and reverse primers. Approximately 101 bp PCR amplicon was generated and used as insert for the cloning experiment. Lane 5= fake fetal DNA, lane 6= fake maternal DNA both at critical Td of 80 °C with D21S1890 internal forward and reverse primers. Positive water contamination clearly seen in NTC lanes

Table 6: Assessment of DNA quality and quantity for PCR purification of fake fetal and fake maternal DNA at Td of 95°C and 80°C.

Samples		Concentration	A260/A280 ratio	A260/A230 ratio
Fake fetal DNA at Td of 95 °C	Band 1	6.6 ng/μl	3.220	0.019
	Band 2	2.4 ng/μl	0.757	0.007
Fake maternal DNA at a Td of 95 °C	Band 1	2.0 ng/μl	0.609	0.005
	Band 2	5 ng/μl	0.833	0.011
Fake fetal DNA at critical Td of 80 °C	Band 1	11.7 ng/μl	4.588	0.010
	Band 2	5.7 ng/μl	1.754	0.009
Fake maternal DNA at a critical Td of 80 °C	Band 1	4.4 ng/μl	2.289	0.027
	Band 2	6.0 ng/μl	0.656	0.027

First attempt was to clone the PCR product of fake maternal DNA and female genomic DNA (with external primers, at a denaturation temperature of 95°C). The Rapid One Shot[®] Chemical Transformation protocol was followed according to manufacturer's instructions. This is an alternative protocol recommended to obtain transformant as quickly as possible and it is also recommended for transformations using ampicillin selection. Thus after performing the TOPO[®] Cloning Reaction (Method 2.7.4), the Cloning Reaction was directly added into two vial of One Shot Chemically Competent *E.coli*, and after five minutes of incubation the mixture were spread on a prewarmed LB plate containing ampicillin and X-gal and incubated overnight at 37°C. The plates showed no colonies for both templates the following day.

When this attempt failed, the same PCR products were used but this time trying the One Shot Chemical Transformation Protocol, which includes heat shocking the cells for thirty seconds at 42°C and adding S.O.C medium. So samples from the first attempt were used (fake fetal DNA and female genomic DNA with external primers at a denaturation temperature of 95 °C) in addition to fake fetal DNA sample with internal primer, at a critical denaturation temperature of 80 °C. After 24 hour overnight incubation, the plates produced blue/white colonies.

Even though blue/white screening can be used to determine if inserts are present, colony PCR was performed to screen for plasmid inserts. Nine colonies were picked at random for colony PCR for each template (fake fetal DNA, female genomic DNA at a denaturation temperature of 95 °C with external primers and fake fetal DNA at critical denaturation temperature of 80 °C with D21S1890 internal primers). Agarose gel result showed no product for all of templates (data not shown), indicating the likelihood that the plasmids were without inserts.

Next attempt was repeating the cloning experiment but with the following changes to the protocol:

- ❖ Performing PCR with BioMix™ DNA polymerase instead of TaqMan polymerase enzyme.
- ❖ Final extension step of PCR condition was changed to ten minutes instead of twenty minutes (Method 2.4.6).
- ❖ Instead of performing gel purification for the PCR product, cleaning of the PCR product was done using QG buffer and isopropanol (Method 2.8.1).
- ❖ For the TOPO® Cloning Reaction 1 µl of fresh PCR product was used instead of 4 µl.

For performing a PCR, female genomic DNA and fake maternal DNA were used as templates and the PCR was performed with a denaturation temperature of 95 °C. Figure 16 shows the result of agarose gel electrophoresis of this PCR, as it shown there is single band for each template around 229 bp; however the marker was not clear. Water contamination observed in lane 3.

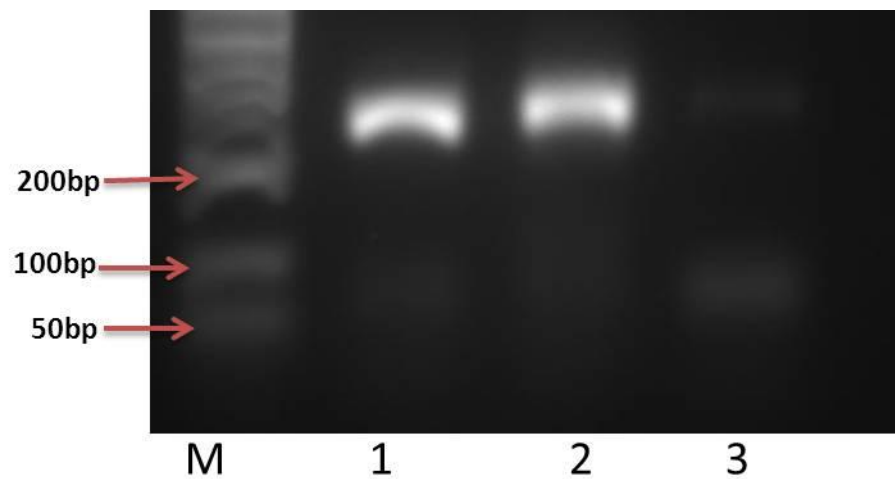


Figure 16: Agarose gel of PCR for female gDNA and fake maternal DNA at a Td of 95 °C. D21S1890 external forward and reverse primers were used to generate PCR amplicons to be used as inserts for the cloning experiment. M= DNA ladder, lane 1= female gDNA, lane 2= fake maternal DNA and lane 3= NTC

Once DNA had been cleaned it was quantified using the NanoVue Plus spectrophotometer (GE Healthcare). Concentrations were calculated from absorbance readings and are presented in Table7.

Table 7: Assessment of DNA quality and quantity for PCR cleaning of female gDNA and fake maternal DNA at Td of 95 °C.

sample	Concentration	A260/A280 ratio	A260/A230 ratio
Female genomic DNA at a Td 95 °C	20.5ng/μl	1.708	0.061
Fake maternal DNA at a Td 95 °C	20.0ng/l	1.674	0.325

After spreading the transformation on a prewarmed plate (containing ampicillin and X-gal), plates were incubated overnight at 37 °C. The next day the plates showed blue/white colonies for only one template (Female genomic DNA) and no colonies for the other template (fake maternal DNA). Five white colonies were picked from the plates for female genomic template, plus one blue colony for colony PCR. This was done to double check if we were getting the right insertion from this PCR product because no colonies were seen with the other PCR product, before carrying out sequencing.

Figure 17 shows product with expected size of ~330 bp for white colonies (229 bp PCR product size + 100 bp vector sequence including M13 primer sequences). This result indicated the plasmids contained the right inserts, however the gel photo shows unexpected product from blue colony which is larger than 100 bp. Theoretically the blue colony is without insert; hence we expected to see a product with 100 bp size (just vector sequence without 229 bp PCR product).

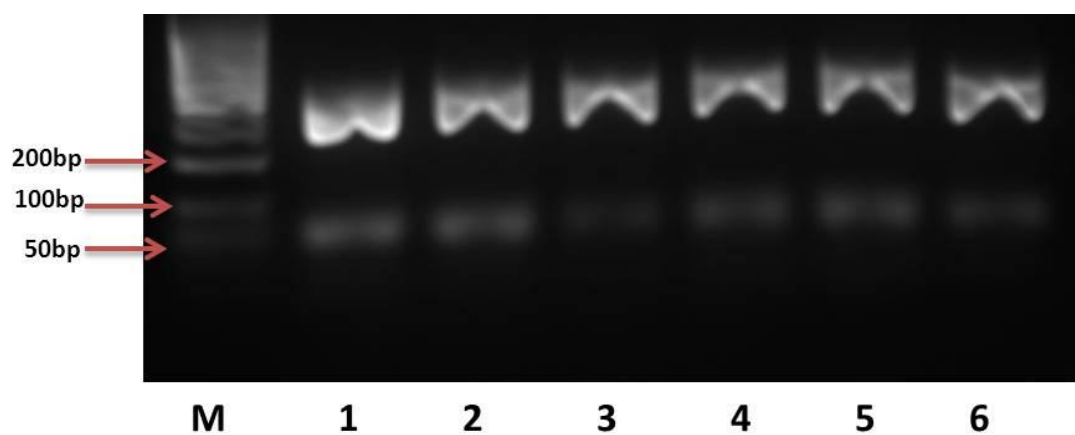


Figure 17: Agarose gel of colony PCR to assess presence of inserts in vector. Female gDNA was used as a template for the insert PCR. M13 forward and reverse primers were used for colony PCR, lane 1, 2, 3, 4 and 5= white colonies of female gDNA, lane 6=blue colony

The next step was isolation of plasmid containing the insert from bacterial cells. Single colonies were picked from the plates and were grown overnight in LB medium containing ampicillin. Plasmid DNA was purified using Miniprep Kit after 16 hours incubation (Method 2.7.7). The concentration of five samples was measured using Nano Vue Plus spectrophotometer (GE Healthcare). Concentrations were calculated from absorbance reading and are presented in Table 8. Finally, all five miniprep samples were sent for sequencing.

Table 8: Assessment of DNA quality and quantity for the five miniprep samples of female gDNA.

Samples	Concentration	A260/A280 ratio	A260/A230 ratio
Sample 1	77.5 ng/μl	2.067	2.460
Sample 2	85.05 ng/μl	2.099	2.881
Sample 3	109.05 ng/μl	2.019	2.449
Sample 4	84.05 ng/μl	2.112	2.561
Sample 5	90.5 ng/μl	2.080	2.919

The sequencing results for the starting template of female genomic DNA were aligned with the reference chromosome (chromosome 21, Appendix A). the result showed the different number of CA repeats for each of miniprep, as presented in Figure 18.

[illegible]

Figure 18: Multiple alignment of DNA sequence (chromosome 21, D21S1890) for female genomic DNA sample at a denaturation temperature of 95°C. M13 forward primers used for sequencing five random white colonies, the result of sequencing from different colonies were compared with D21S1890 product with external primers. The yellow sections indicate D21S1890 external forward and reverse primers; the green section indicates different number of CA repeats between each sample. Base pair T (highlighted in red) most probably due to sequencing error.

To test for plasmid DNA with the correct insert and also to see how the CA repeats looked on an acrylamide gel, purified DNA was digested with EcoRI (Method 2.8.8). The resulting DNA fragments were separated on acrylamide gel. The acrylamide gel electrophoresis results showed bands within the expected size (229 bp) (Figure 19), also the results showed that two of the samples (Lane 3 and 5) showed same product size, sequencing results of these two samples showed same number of CA repeat (20) as shown in Figure 19 1F and 5F.

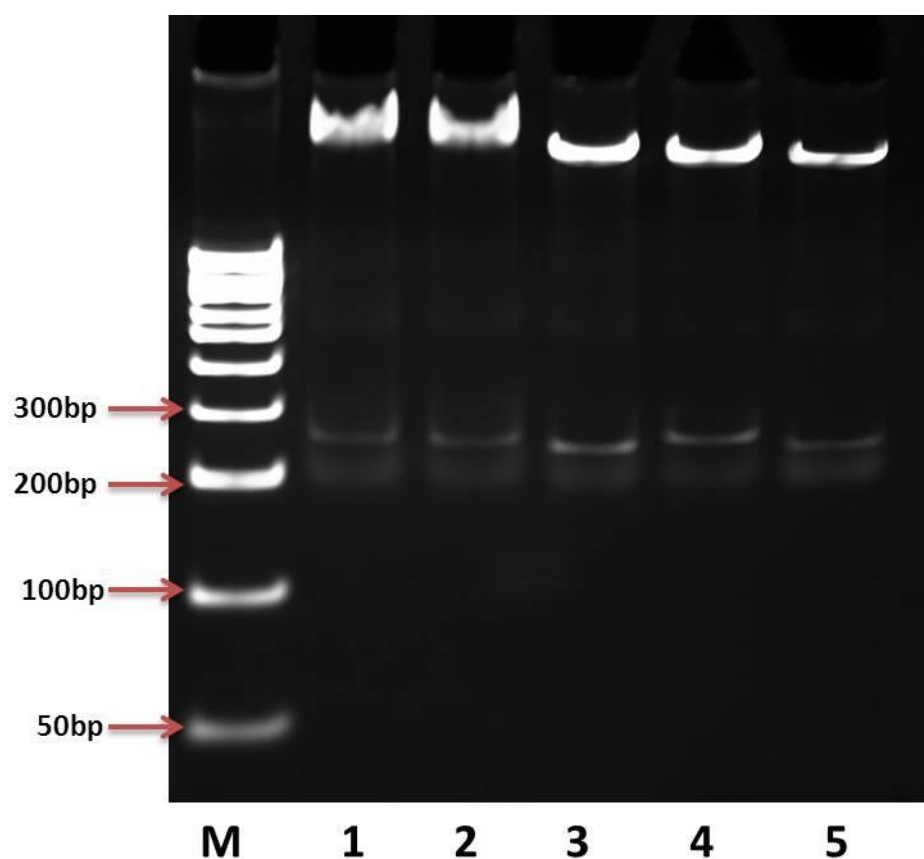


Figure 19: Non denaturing polyacrylamide gel for the restriction enzyme to confirm the insert of female gDNA in pCRTM4-TOPO® TA vector. 10% acrylamide gel stained with Gel Red was used for five white colony samples of female gDNA. M= DNA ladder, lane1, 2, 3, 4 and 5 different white colonies of female gDNA template.

The next step was to repeat the running of a PCR for the fake maternal DNA template. Therefore PCR was performed with D21S1890 external forward and reverse primers using BioMix DNA polymerase at a denaturation temperature of 95°C (Method 2.3.6). A portion of the PCR product was initially run across an agarose gel. This was to verify that product had been amplified and that it was of the correct size. The gel photo showed that the template had given a faint band at approximately 229 bp as expected as shown in Appendix L. Then cleaning of the product was done (Method 2.7.1). Once the DNA had been cleaned, it was quantified using the NanoVue Plus spectrophotometer (GE Healthcare). Concentrations were calculated from absorbance readings: the concentration was 12.1ng/μl and the A260/A280 ratio was 1.806, and the A260/A230 ratio was 0.025. TOPO[®] Cloning Reaction and transforming to *E.coli* cells was done same as before. The plates did not show any colonies for the first twenty four hours, but after forty eight hours the plates showed blue/white colonies. However, the white colonies were smaller and fewer in number than the blue colonies. Colony PCR was done for ten of the white colonies. Figure 20 indicates that the plasmids were without the inserts.

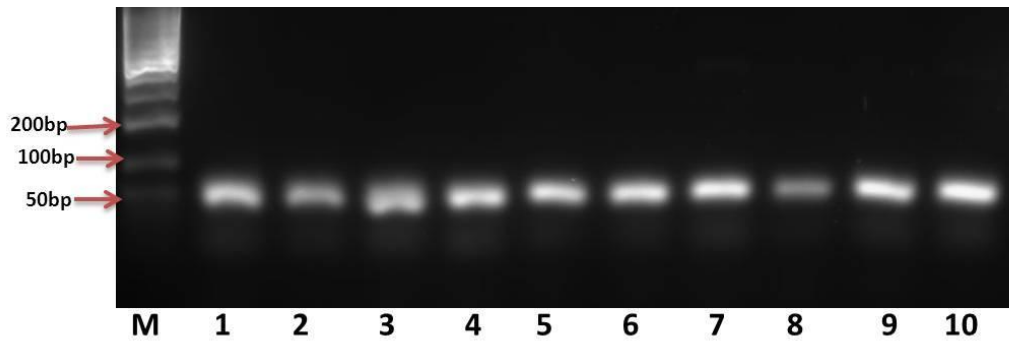


Figure 20: Agarose gel of colony PCR for fake maternal DNA (6534H). D21S1890 external forward and reverse primers were used to generate PCR amplicons to be used as inserts for the cloning experiment. M13 forward and reverse primers were used for colony PCR, M= DNA ladder, lanes 1-10 are different white colonies of fake maternal DNA.

With limited time to complete the project, it was not possible to order a new cloning kit for repeating the cloning experiment. A PhD student Kelly Sillence (Plymouth University) was able to repeat the cloning experiment using a new cloning kit but with same fake fetal DNA sample. Therefore the cloning experiment was repeated for fake fetal DNA template at denaturation temperatures of 95 °C and 80 °C. The sequencing results showed no sequencing for the CA repeat region at the critical denaturation temperature of 80 °C for fake fetal DNA sample (the result is presented in Appendix M). However this result shows drop out of more than just the CA repeat region at a critical denaturation temperature of 80 °C. The sequencing was done for two white colonies chosen at random, so sequencing of more colonies at critical denaturation temperature of 80 °C would maybe more informative.

4 Chapter Four

4.1 Discussion

Testing for birth defects and inherited diseases during pregnancy first became possible in the late 1960's with the development of lab techniques that were able to grow fetal cells found in amniotic fluid. The foremost reason that pregnant women decide to enter any kind of prenatal screening programme is for the detection of aneuploidy, in particularly Down syndrome, which is the most common cytogenetic anomaly in live births. Other fetal aneuploidies frequently detected involve chromosomes 13 (Patau syndrome) or 18 (Edwards syndrome). Once the chromosomal abnormality responsible for the phenotypic appearance of Down syndrome had been identified, it became possible to make a firm diagnosis based on cytogenetic studies (Maliszewski, 2010),

Currently, prenatal diagnosis of aneuploidies relies on invasive procedures such as CVS in the first trimester and amniocentesis in the second trimester. Following these procedures, the full fetal karyotype is usually determined using cultured cells. The two weeks period needed for cultivation and subsequent analysis has proven to be associated with considerable parental anxiety and medical problems in those situations requiring therapeutic intervention. The disadvantages of CVS and amniocentesis are that they involve introducing a needle into the uterus, which is associated with the risk of morbidity and mortality and transmission of infection to the fetus at the time of the invasive procedure (Giorlandino et al., 1994).

It is because of these procedure-related risks, and how they are assessed against the value of the information obtained, that many women opt to refuse having prenatal diagnostic testing. For some women, despite wanting the information or considering it valuable, they are simply not willing to risk miscarriage. If a woman chooses not to have diagnostic testing, the most knowledge she can gain is through combining ultrasound findings with maternal serum screening. Miscarriage is not associated with either of these screening methods; however they are not diagnostic tests.

As the invasive procedures bear a considerable risk for fetal loss, a long sought goal in prenatal medicine is the establishment of non-invasive tests that allow for prenatal genetic testing. The traditional strategy to accomplish this goal was focused on fetal nucleated cells found in the maternal circulation (Herzenberg et al., 1979, Lo et al., 1997). However a number of obstacles including their persistence in maternal circulation after pregnancy, rarity and lack of fetal specific markers, meant that another approach needed to be found (Bianchi et al., 1996, Avent et al., 2008). The later discovery of cffDNA in maternal plasma (Lo et al., 1997) has shifted the attention towards this target. cffDNA disappears rapidly from the maternal circulation postpartum (Lo et al., 1999), this means that it is pregnancy specific and would not lead to false positive results in future pregnancies.

The developing possibilities of NIPD may lead to significant changes in the testing and screening of pregnancies. Research on non-invasive testing began by examining the placental barrier between mother and fetus. The result obtained indicated that the fetus could release its DNA and RNA into the mother's circulation. So initially, interest was focused on genes, gene products and/or mutations that are passed on to the fetus by the father and which were distinguishable from those of the mother. Although some work has been carried out using fetal cells obtained from the cervical mucus (Mantzaris et al., 2005) or fetal DNA in maternal urine (Shekhtman et al., 2009).

Most research has been focused on strategies to detect cffDNA from the fetus in the maternal circulation. A number of clinical applications of cffDNA analysis for prenatal screening and/or diagnosis have been developed, based on distinct and detectable differences between fetal and maternal genome like fetal sex or fetal Rhesus D status. Fetal sex determination is feasible and reliable using cffDNA from 7 weeks of gestation through the detection of sequences on the Y chromosome and has been available to all women at risk of X-linked disorders (Costa et al., 2002, Finning et al., 2002, Avent and Chitty, 2006), and congenital adrenal hyperplasia (Rijnders et al., 2004). It has been estimated that this application may reduce the need for invasive testing by 50% (Finning and Chitty, 2008). A study by Hill et al. (2011) investigating the NHS costs of NIPD and invasive prenatal diagnosis, showed that the costs of NIPD and invasive prenatal tests are similar for fetal sex determination. Hence, NIPD can provide benefits for many women by avoiding the risks of invasive testing, without acquiring additional costs.

In addition fetal RhD status determination has widely been used in pregnancies involving RhD-negative women (Lo et al., 1998, Finning et al., 2004, Scheffer et al., 2011). Sbarsi et al. (2011) demonstrated accurate procedures for determining of fetal RHD genotype, thus permitting early diagnosis of at risk pregnancies and the correct management of HDFN. This enables unnecessary treatments to be avoided, and prevents wastage of anti-D immunoglobulins.

NIPD is limited by the minimal quantity of fetal sample available for analysis and thus requires the application of new technologies with extremely high sensitivity. The problem with detecting cffDNA is that it appears in low concentration against a background of maternal DNA (Chan et al., 2004, Li et al., 2004b).

RT-PCR had been employed as a conventional tool to amplify cffDNA, aiming to enrich cffDNA and ultimately to be able to detect aneuploidies. The downside of this method is that it does not just amplify cffDNA but also maternal DNA. This raises the need for distinguishing features between the DNA of mother and baby. Therefore, COLD-PCR was tried as an alternative method to enrich for cffDNA against a maternal background.

In this study we have attempted to test the theory that COLD-PCR could be used to selectively amplify fetal DNA. The denaturation temperature of cffDNA is predicted to be lower due to its smaller fragment size, and this might mean it could be selectively amplified with COLD-PCR. This investigation focused on a fragment on chromosome 21 containing a CA repeat STR, identified as D21S1890.

Fake fetal DNA was generated from female genomic DNA (Promega) using D21S1890 external forward and reverse primers. 6534H genomic DNA (from buffy coat) was regarded as fake maternal DNA. This was selected from twenty four genomic DNA samples after comparing it with female genomic DNA sample, in which the results from fragment analysis showed matching and distinguishing peaks between them, as shown in Figure 5 D.

The first step was trying to find the critical denaturation temperature, at which fake fetal DNA would denature while fake maternal DNA would remain double stranded and not amplified. Hence enrichment of the fetal DNA fragment can then be used for simple methods of detecting trisomy such digital PCR. The only limitation with methods such as digital PCR is the low concentration of fetal DNA in the maternal circulation, thus prior enrichment of the fetal DNA is required for these methods.

Early experiments tried to repeat the same critical denaturation temperature that had been identified before (unpublished work in Neil Avent's lab). The previous work was done using end point, conventional PCR and then running samples on an agarose gel to check for the product size. The previous results determined that 78.8 °C was the critical denaturation temperature. However the results from running RT-PCR with the identified critical denaturation temperature (78.8 °C), using fake fetal DNA were negative i.e. no amplification of the DNA templates (fake fetal DNA and female genomic DNA) (Appendix C1), which meant that this temperature was too low even to amplify fake fetal DNA.

Therefore, the next step was to test different denaturation temperatures in an attempt to find the critical temperature for the selective amplification of fetal DNA. A denaturation temperature of 79.4 °C was also tried but again the result showed no amplification of fake fetal DNA. A range of denaturation temperatures between 84 °C and 81°C were tested. At 84 °C the results showed amplification of both fetal and genomic DNA with mean Ct value of 30 for fetal DNA however the genomic DNA was undetermined but the fetal DNA showed amplification at very late cycles as shown in Figure 4 B. At denaturation temperature of 83 °C the mean Ct value for fake fetal DNA was 32, so by getting the denaturation temperature lowered to 82°C we were getting amplification of just fake fetal DNA but at the late cycles.

So another range of temperatures were tested to find the temperature at which fake fetal DNA would amplify at an earlier cycle but with no amplification of genomic DNA at the same temperature. Temperatures of 81.8 °C- 81.6 °C- 81.4 °C- 81.2 °C and 80 °C were tested for this reason. At 81.6 °C and 81.4°C the results showed amplification of both fake fetal DNA and genomic DNA however the genomic DNA template were amplifying at very late cycles. RT-PCR at a denaturation temperature of 81.2 °C showed mean Ct value for fake fetal DNA of 22 and undetermined Ct value for female genomic DNA. However this PCR run showed contamination in all NTC replicates, despite of repetition of the PCR run, therefore this temperature was not identified as the critical denaturation temperature (Appendix C2).

Thus identifying 80 °C as critical denaturation temperature for this study was based on the following: getting amplification of fake fetal DNA at early cycles without amplification of genomic DNA, mean Ct value for fake fetal DNA was 29 (Figure 4 C).

Since the fake fetal DNA was generated from female genomic DNA, fake maternal DNA was needed for establishing the spike experiment which required making a sample of mixed composition of fake maternal and fetal DNA that imitated the blood sample of a pregnant woman, which is a mixture of fetal and maternal DNA. As mentioned before different genomic DNA samples from buffy coat were tested in an attempt to find fake maternal DNA. Fragment analysis was done for all these genomic DNA samples, and female genomic DNA was compared with each of these samples depending on the fact that every person will have two alleles: one from mother and the other from father so ideally we should have two peaks for the fake fetal DNA and for identifying fake maternal DNA we should find a sample with one peak matching fetal peaks and another not matching fetal peaks see (Figure 21).

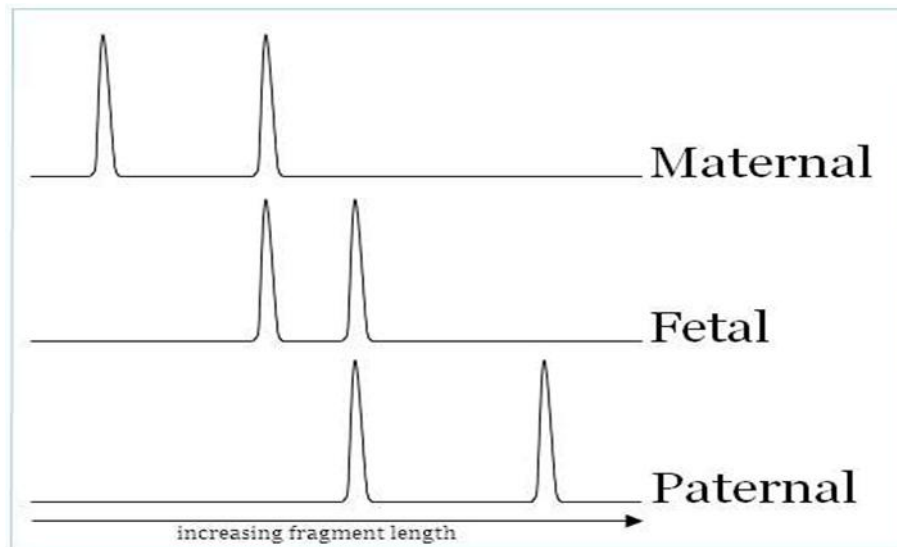


Figure 21: Schematic diagram showing the fragment length of alleles from a heterozygous fetus along with the alleles of the mother and father (both also heterozygous). This explains why, when looking for a fake fetal and maternal pair, the pair should ideally have a matching and distinguishing allele. This diagram also shows the fragment lengths as they should appear: without stutter bands.

The peaks representing allele size are separated by multiples of two base pairs, as expected for a CA STR sequence. However, as each individual has only two alleles the observed result of multiple peaks was not expected. Despite the stutter peaks in the fragment analysis result, the genomic DNA sample (6534H) was identified for use as fake maternal DNA. A stutter peak was one of the main issues that were highlighted in this study.

Stutter peaks

Stutter products are regarded as a biological artefact of STR marker analysis, which makes mixture analysis more difficult. Stutter pattern is less pronounced with larger repeat unit sizes, which means that it occurs more with dinucleotide repeats than with tri- or tetra-nucleotide repeats (Murray et al., 1993). A mechanism known as slipped strand mispairing has been proposed to explain the stutter bands that result from the amplification of dinucleotide repeats. According to this proposal, the template strand and extending strand can spread apart during synthesis through the repeat region perhaps when the DNA polymerase has fallen off during the PCR. A single unit can then loop out in the template strand before the two strands are reannealed. The result is that the newly extended strand will have one fewer repeat unit than the template strand when synthesis is complete (Walsh et al., 1996).

Miller & Yuan,(1997) demonstrated that a major problem of doing quantitative analysis of genomic STR is the stutter pattern. They proposed that stutter patterns may arise from factors such as Taq Polymerase misreading or secondary structure of DNA.

Kellogg et al. (1994) explained that stutter patterns can be reduced, and sometimes eliminated, by optimizing PCR conditions using different primers and using hot-start PCR. They showed that the change in size distribution with increasing cycle count implies that the stutter is due to Taq Polymerase skipping or gaining a dinucleotide repeat, rather than arising from DNA secondary structure and the explanation is because if the stutter were due to secondary structure, the pattern would be expected to remain constant during amplification.

Moretti et al.(1998) demonstrated that the AmpliTaq Gold DNA polymerase is a thermostable enzyme which effectively simulates (hot start) PCR in a fast, simple and practical fashion. The use of this enzyme can reduce or eliminate the generation of non-specific PCR products that can result from mispriming and primer oligomerization with the use of some primers and substandard reaction conditions. This group clarified that the undesired PCR products compete with targeted sequences for dNTPs and primers and, as a result, reduce the yield of specific target products. They assessed the usefulness of AmpliTaq Gold DNA polymerase in a multiplex STR amplification system that exhibited a non-specific PCR product and poor yield at two of the four loci when AmpliTaq DNA polymerase was used (Forslund et al., 1999, Thompson et al., 1998).

In the spike experiment, we have imitated the environment for cffDNA during early and late pregnancy by having different mixtures of fake fetal and fake maternal DNA. For the early pregnancy the simulation we used was 5% of fake fetal DNA, while for the late pregnancy we used 10%. A study by (Lo et al., 1998) showed the concentration of cffDNA to be 3.4% and 6.2% of the total plasma DNA in early and late pregnancy respectively. So we were broadly trying to establish differences between first and third trimesters.

RT-PCR results of spike experiment clearly showed amplification of fake fetal DNA without amplification of fake maternal DNA at the critical denaturation temperature (80°C). However running the same templates under the same conditions but with D21S1890 FAM labelled internal forward and reverse primers (without using D21S1890 probe) on conventional/end point PCR, the agarose gel showed a product at 80 °C critical denaturation temperature for the fake maternal DNA. In addition the results also showed larger product than the expected size (101 bp) for fake maternal DNA. This was another problem that was highlighted for this study.

The mix template used for the spike experiment was a 90:10 mix of maternal and fetal fragment respectively. However fetal fragment was purely D21S1890 whereas maternal was genomic so there would actually have been more D21S1890 from fetal than maternal. This might explain why the electropherogram of the mix template corresponded strongly with that of the fetal but not of the maternal; when PCR was ran with a Td of 95°C the mix was expected to correlate more strongly with the maternal but this was not the case (Figure 12 A and B).

RT-PCR quantification method has many advantages over the conventional quantifications in terms of accuracy, sensitivity, dynamic range, high-throughput capacity, and absence of post-PCR manipulations. Sequence-specific fluorescence-labelled probes (e.g. TaqMan) have been considered as a standard detection format in many diagnostic and research applications, but are not very well suited for quantification of a large number of different sequences, because a new and relatively expensive probe is generally required for each amplicon under investigation (Freeman et al., 1999, Ponchel et al., 2003, Heid et al., 1996).

Analysis of spiking products on an acrylamide gel (Figure 11) also suggested that the size of amplicon resulting from the fake maternal DNA template was larger than expected. Running spiking experiment on genetic analyser for fragment analysis also showed decreased number of maternal peaks at a critical denaturation temperature of 80 °C (i.e. not complete absence of fake maternal DNA peaks) (Figure 12).

SYBR Green for melt curve analysis was performed to confirm if we were getting single specific products, and also to check if we were getting the same melting temperatures for templates at denaturation temperatures of 95 °C and 80 °C. Melt curve analysis was carried out for spiking experiment products using gradient options on StepOnePlus software, at denaturation temperatures of 95 °C and 80 °C. Nevertheless SYBR Green based RT-PCR is less specific (detects all double stranded DNA including primer-dimers and other undesired products) so the chance of getting heteroduplex is more when there is reduction in the specificity (Thompson et al., 2002).

The amplification plot of fake maternal DNA with SYBR Green assay at a critical denaturation temperature of 80°C in Figure 13 B indicates possible heteroduplex formation, as the same fragments showed no amplification at the same temperature with TaqMan mastermix and probe. Melt curve analysis result showed different melting temperature between fake fetal DNA and spike sample at a critical denaturation temperature of 80 °C (Figure 14), in addition to shoulder peaks for all templates at the same critical denaturation temperature. These peaks could have been generated also by **heteroduplex** formation.

Genomic DNA exists in the form of homoduplexes, with all corresponding base pairs being complementary, Adenine: Thymine (A: T) and Cytosine: Guanine (C: G). Heteroduplexes occur when dsDNA contains noncomplementary base pairs. Heteroduplexes arise during the plateau stage when dsDNA is more likely to be formed by hybridization than extension. (Palais, 2007a).

Upchurch, Shankarappa & Mullins (2000) has defined heteroduplexes as DNA molecules which have mismatched or unpaired bases. These heteroduplexes can be formed *in vivo* in several ways, such as by replication errors, during genetic recombination between similar but not identical DNA strands, or by spontaneous or chemical modification of bases. The heteroduplexes are more structurally unstable than the homoduplexes due to mismatches and unpaired nucleotides. These structural differences cause heteroduplexes to have reduced mobilities compared to those of the homoduplexes. The resulting reduction in band mobility and peak mobility can be detected by polyacrylamide gel electrophoresis and capillary electrophoresis, respectively.

Homoduplex molecules migrate faster through the gel in comparison to the heteroduplex molecules, which move at a slower rate and often produce a second band below the amplification product (White and Kuskawa, 1997). This can be observed in Figure 6 B in this study. Janse, Bok & Zwart (2004) identified that a longer final elongation period and a reduction in the number of PCR cycles can help to reduce the identification of double bands in gel electrophoresis.

Heteroduplexes and homoduplexes can be differentiated by temperature gradient capillary electrophoresis (TGCE) by heating the amplicons so they partially denature during migration through capillaries. Heteroduplexes denature at a lower temperature and migrate more slowly than homoduplexes during capillary electrophoresis. A homoduplex generates only one TGCE Peak, while heteroduplexed samples can yield up to four peaks (Palais, 2007b).

Some heteroduplexes can be eliminated from PCR products by using a single strand cleaving endonuclease to resolve internal single strand loop structures. Single stranded loop structure can form because heteroduplexes migrate anomalously during gel electrophoresis forming additional bands on agarose gels, however this can be eliminated or removed by gel purification of the desired PCR product (Qiu et al., 2001).

A study by Thompson et al. (2002) demonstrated a reconditioning PCR method to eliminate heteroduplexes which was based on the principle that formation of homoduplex DNA will be favoured to the exclusion of heteroduplex DNA in the presence of excess primer. By restoring the initial primer concentrations during the reconditioning PCR a denatured DNA molecule will have a higher probability of annealing with a primer than with a heterolog, leading to extension of homoduplex. By the same principle, the formation of PCR chimeras from annealing and extension of heterologous DNA fragments can be reduced by optimizing the number of amplification cycles to maintain an excess of primer through the end point of reaction.

Throughout the experiments, an indication of **primer dimer** formation has been apparent. Primer dimers are routinely observed in PCRs. They are not derived from template DNA and they can complicate experimental analysis. In the PCR, template-independent primer interactions can take place that give rise to non-specific products, notably primer dimers; this can be seen clearly in Figure 6 A (lane 4 and 5). Because primers are present at high concentrations, weak interactions can occur between them. Primer dimer formation can be reduced by careful primer design, the application of stringent conditions, the use of 'hot-start' and enzyme formulations such as AmpliTaq Gold (Brownie et al., 1997).

Alternatively **PCR contamination**, which is a common problem in molecular laboratories, can contribute to the amplification of undesired products in PCR assay. Another problem that we have faced throughout the experiment was regarding the fake fetal and maternal DNA sample (generated from female genomic DNA) as these samples were freeze- thawed many times during the period of research. Therefore heteroduplex formation, contamination and CA repeats could be all regarded as a reason for stutter peaks.

By sequencing these samples it was hoped we would be able to explain these secondary products and heteroduplex formation. Thus cloning was the next attempt to solve the problem, and also only by cloning and then sequencing we would be able to determine the different STR repeats for the samples. Our intention was to clone the fake fetal DNA at both 95°C and 80°C denaturation temperatures so that we would be able to sequence and then prove if there is any sequence that is being deleted by carrying out PCR at a lower denaturation temperature.

Our goal was to clone both templates (fake fetal and fake maternal DNA). However we were able to clone female genomic DNA from which fake fetal DNA was produced from; but unfortunately the cloning experiment was not conclusive for sequencing these samples as we found out later that female genomic DNA sample was from anonymous pooled female blood samples.

Due to the limited time available, it was not possible to repeat the cloning experiment for the other template (fake maternal DNA) or using new cloning kit to repeat the entire cloning experiment. However the result of sequencing fake fetal DNA sample at denaturation temperatures of 95 °C and 80 °C, by Kelly Sillence, clearly showed no sequence of CA repeat region for the fake fetal DNA at a critical denaturation temperature of 80 °C. While at a denaturation temperature of 95 °C the result showed sequencing of the CA repeat region. This result shows that there is the possibility of hairpin loop formation, resulting in looping out, which is more likely to have occurred at the lower denaturation temperature.

The hairpin-loop is a secondary structural motif frequently observed in both DNA and RNA where there is self-complementarity in the sequence. DNA hairpin-loops are involved in various biological functions. McMurray (1999) explained that polymerase slippage occurs at repeats because directly repeated sequences can provide multiple sites for pairing of a complementary strand if the duplex becomes unpaired. However, slippage to a matching repeat tends to be small. This is because slippage by more than a few repeating units becomes energetically unfavourable, because many more bonds must be broken in the template than are reformed at a loop.

COLD-PCR seems the most encouraging enrichment technique discussed but requires further investigation. The goal for selective cffDNA enrichment is to allow its use in NIPD for Down syndrome. So after identification of critical denaturation temperature (80°C) for samples that contain primers designed for chromosome 21 marker, also alternative primers will need to be designed for a reference chromosome (often 13, 18 or X) so that they can be run on RT-PCR. When fluorescent dyes or probes are incorporated into the products of PCR amplification, STR markers can be rapidly analysed and quantified using DNA sequencer analysis, which can lead to the identification of allele ratios and therefore subsequent diagnosis of trisomy 21 (Adinolfi et al., 1997, Lo et al., 2007a). This method would be a lot quicker and cheaper than measuring relative chromosome dose by massively parallel sequencing.

There remains a need therefore for an improved NIPD method, which is not limited in the way that the known method discussed above is constrained. In particular it would be advantageous if the NIPD method could overcome the problems associated with the low fractional concentration of cffDNA present within maternal plasma sample. Therefore, a rapid and accurate molecular based detection of aneuploidy is highly desirable. Digital PCR, a pure quantitative approach, requires minimal sample handling and analytical step. Digital PCR assays are universal and are not dependent on genetic polymorphisms; in contrast, the most common type of QF-PCR requires multiple polymorphic markers. Digital PCR is also superior to FISH in that FISH is labour intensive as mentioned before and requires trained personnel and intact cells for analysis. Digital PCR is regarded as one of the more preferable approaches for the NIPD of Down syndrome.

4.2 Conclusion

Current invasive techniques for the early prenatal diagnosis of DS, such as CVS or amniocentesis, are undesirable as they carry a 1% risk of miscarriage. As compared with the present methods of prenatal diagnosis; NIPD has the advantage of high sensitivity with no risk for miscarriage.

Despite issues with altered size of products in COLD-PCR, this study describes how the use of COLD-PCR can allow for effective amplification of fetal DNA, due to the selective discrimination against larger genomic DNA fragments at identified critical denaturation temperature. A number of issues require further investigation, such as stutter peaks, as ideally there would be no stutter peaks making the true fragment length easier to interpret. The different product sizes at different denaturation temperatures may require redesign of the primers. The use of an alternative polymorphism could be also investigated for example; tri- or tetra-nucleotide STRs. Further research also needs to focus on eliminating heteroduplex formation.

Also it is crucial to validate this project by using actual clinical samples, in this way one will be able to see if the enrichment technique achieved by COLD-PCR is able to be of diagnostic value in non-invasive prenatal medicine. Large scale studies to validate this technique are required, before this method of PCR amplification can be used in conjunction with sequencing analysis for trisomy 21 diagnosis in a clinical setting.

4.3 Future work:

- Perform a larger-scale clinical study which is essential in order to enable the introduction of the new test into clinical practice.
- Provide an accurate, fast, simple and cost effective test that can be safely used in the clinical practice.
- Further development of the new test so it can be utilized for the non-invasive prenatal diagnosis of other syndromes such as trisomy 13, 18 and aneuploidies associated with chromosomes X and Y.
- Development of non-invasive prenatal diagnosis for other diseases and syndromes.

5 Chapter Five

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6 Chapter Six

6.1 Appendix

(B008ZE5, W6165, RH31495, RH73997, HSB008ZE5, SHGC-21170)

Het: 0.90

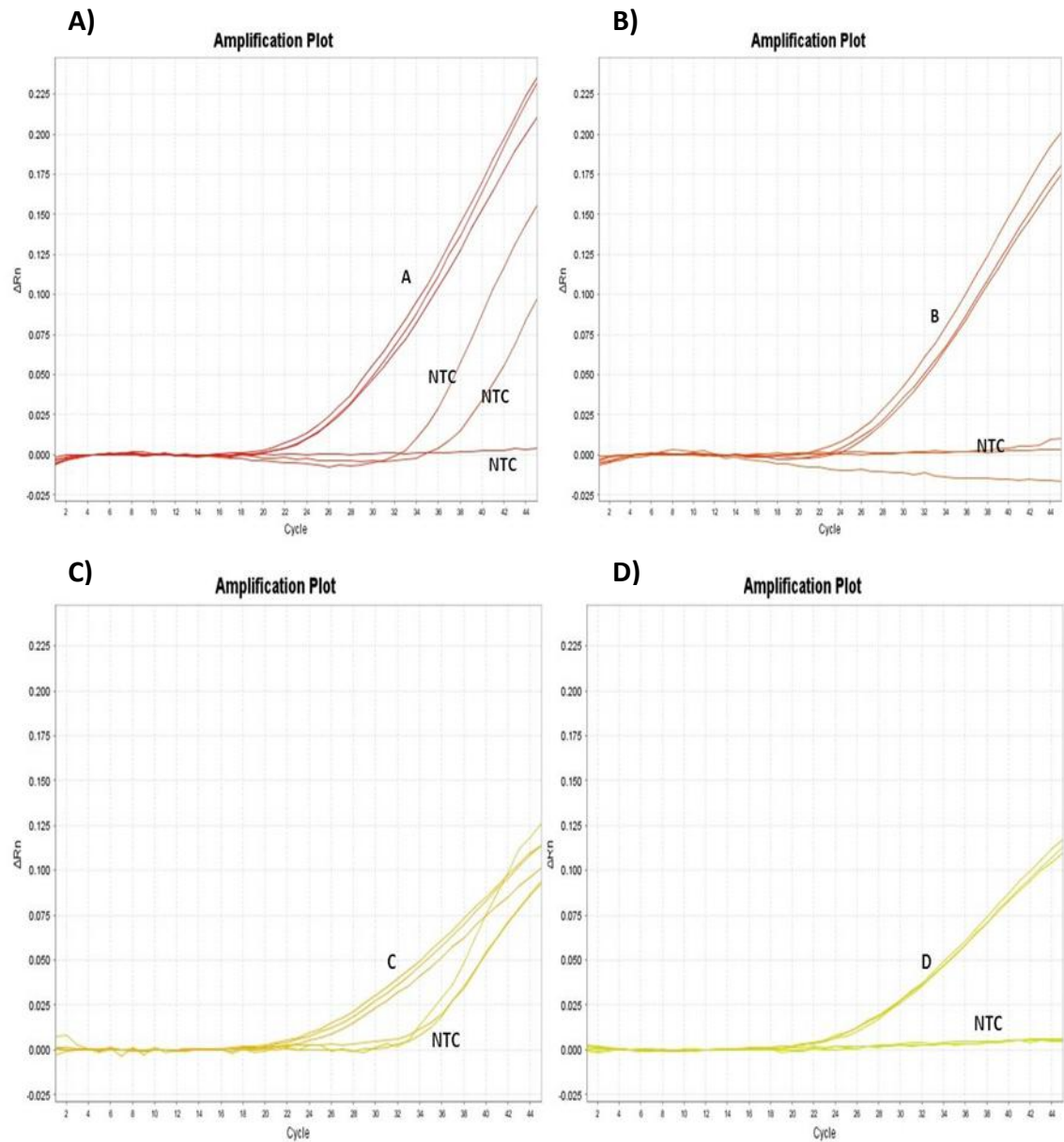
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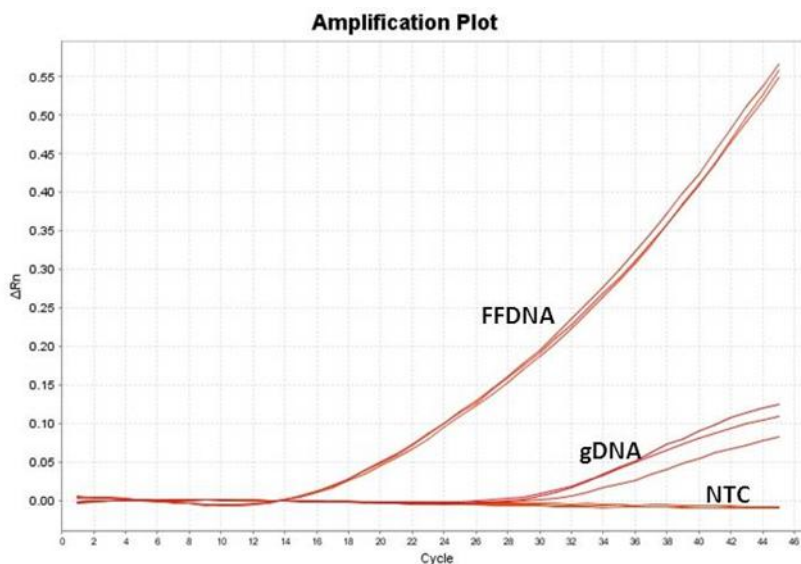
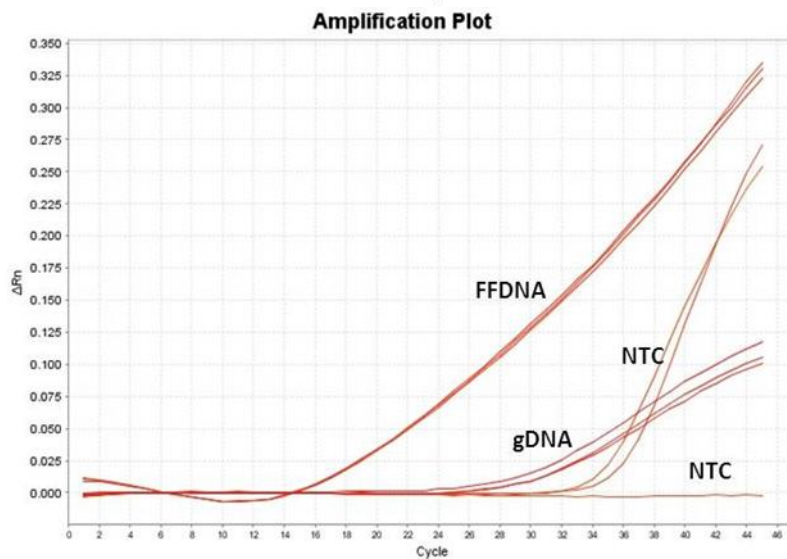
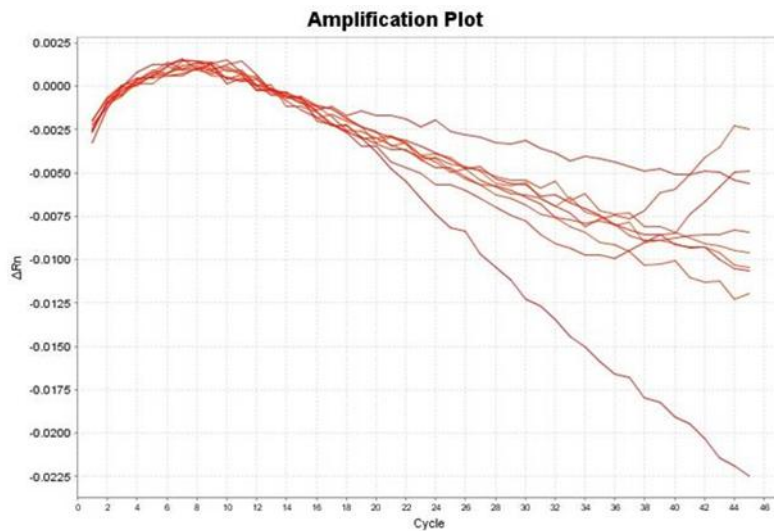
Appendix A: D21S1890 STR, identifying internal primers (blue), external primers (green) and probe (pink), CA repeat region underlined. These primers were primarily used for RT-PCR. Designed by Alice Bruson (a visiting PhD student from Università delgi di Padova, Italy).

D21S1890 External Product = 229bp)

D21S1890 Internal Product = 101bp)



Appendix B: RT-PCR amplification plot at a denaturation temperature 95°C, with different concentrations of D21S1890 primers and probe. Female genomic DNA was used as a template, at a final concentration of 20 ng/μl. **A)** 200nM of primer mix and 100nM of probe showing mean Ct value of 32. **B)** 300nM of primer mix and 100nM of probe showing mean Ct value of 34. **C)** 200nM of primer mix and 50nM of probe showing mean Ct value of 38 and **D)** 300nM of primer mix and 50nM of probe with mean Ct value of 37. NTC shows contamination in amplification plot A and C.

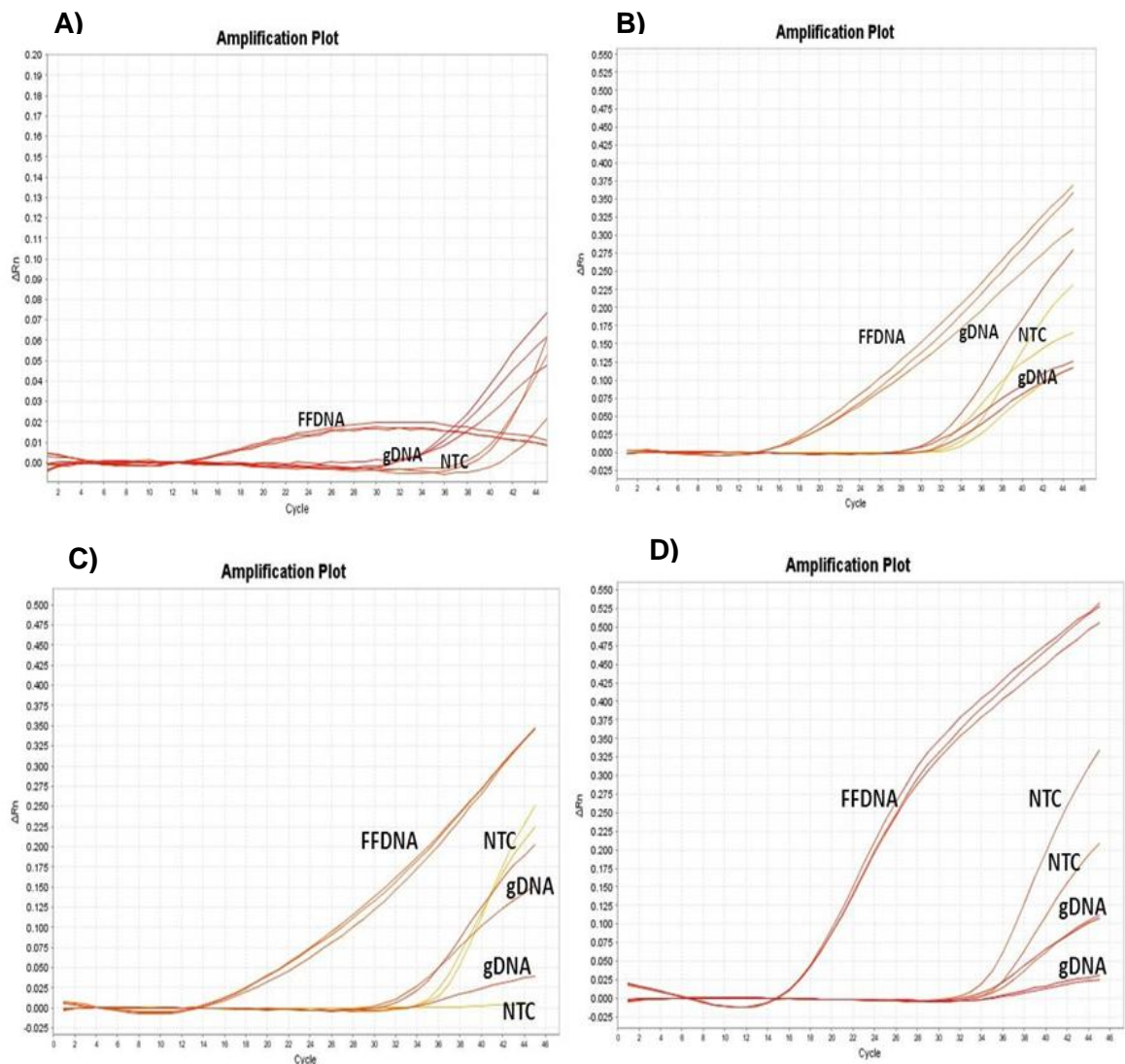


Appendix C1:

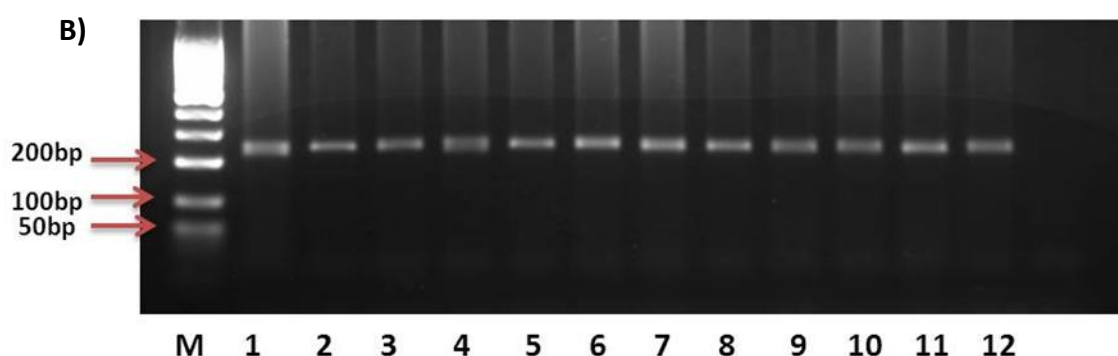
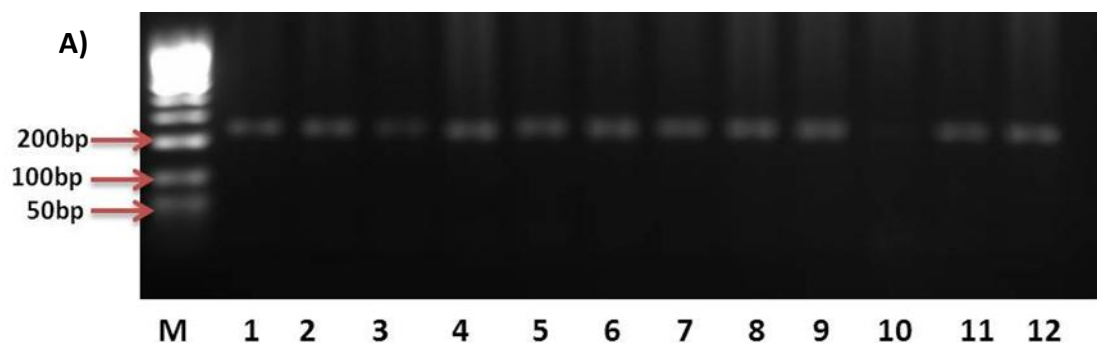
A) RT-PCR amplification plot at a denaturation temperature of 78.8°C.

B) RT-PCR amplification plot at 83°C. Fake fetal DNA (FFDNA) has mean Ct value of 32, undetermined Ct value for gDNA. NTC shows contamination.

C) RT-PCR amplification plot at 82°C. Fake fetal DNA (FFDNA) has mean Ct value of 28, undetermined Ct value for gDNA. NTC shows no contamination



Appendix C2: RT-PCR amplification plots at different denaturation temperatures. **A)** at a denaturation temperature of 81.8°C, both fFDNA and gDNA shows undetermined mean Ct value **B)** at a denaturation temperature of 81.6°C, fFDNA has mean Ct value of 31 and gDNA has mean Ct value of 38 for one replicate and undetermined for other two replicates. **C)** At a denaturation temperature of 81.4°C, fFDNA has mean Ct value of 32 and gDNA has mean Ct value of 42. **D)** At a denaturation temperature of 81.2°C, fFDNA has mean Ct value of 22 and undetermined for gDNA. All amplifications show contamination in NTC lane.



Appendix D: Agarose gel of PCR for identifying fake maternal DNA. PCR was carried out using D21S1890 external forward and reverse primers and 24 gDNA samples from buffy coat as templates.

A) M= DNA ladder

Lane 1= 075D gDNA

Lane 2= 728Q gDNA

Lane 3= 55309 gDNA

Lane 4= 6515N gDNA

Lane 5= 739K gDNA

Lane 6= 6529BgDNA

Lane 7= 427H gDNA

Lane 8= 9604A gDNA

Lane 9= 747K gDNA

Lane 10= 748I gDNA

Lane11= 96242 gDNA

Lane 12= 9670R gDNA

B) M=DNA ladder

Lane 1= 6374 gDNA

Lane 2= 103E gDNA

Lane 3= 524J gDNA

Lane 4= 735S gDNA

Lane 5= 944V gDNA

Lane 6= 753T gDNA

Lane 7= 631E gDNA

Lane 8= 057D gDNA

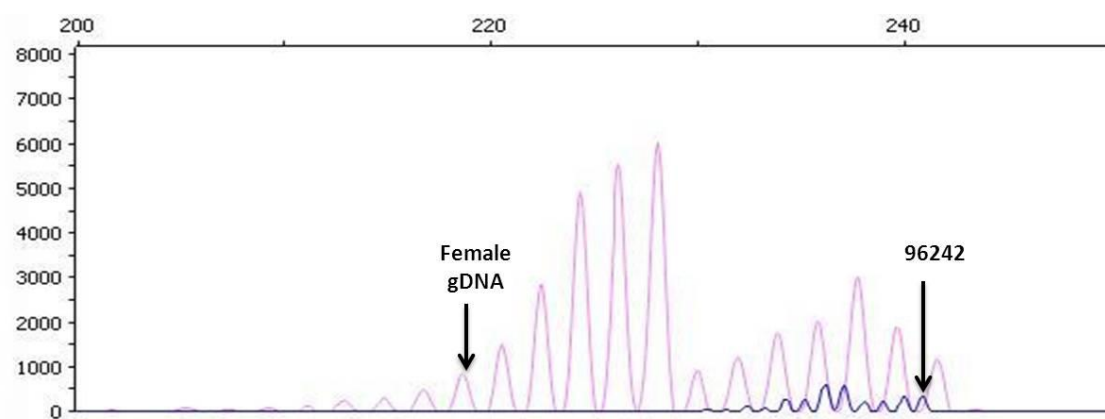
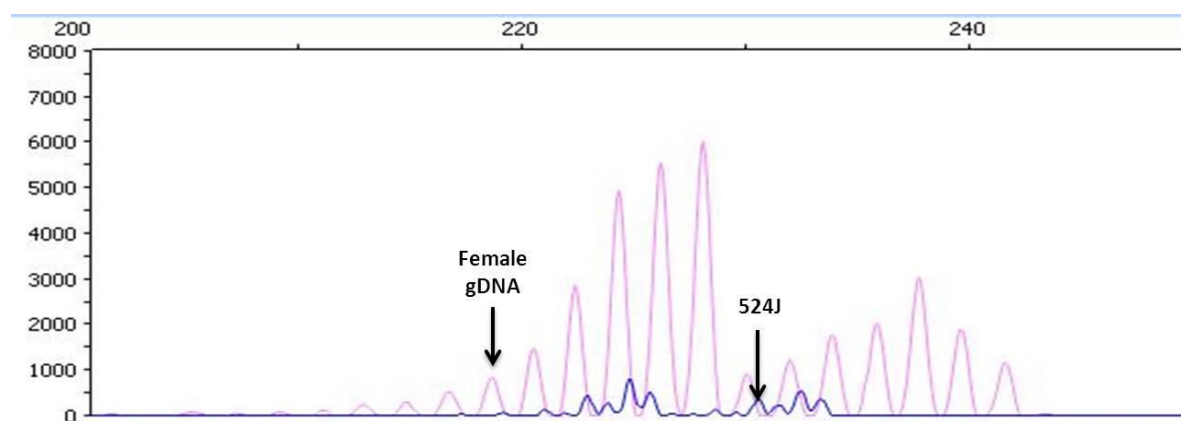
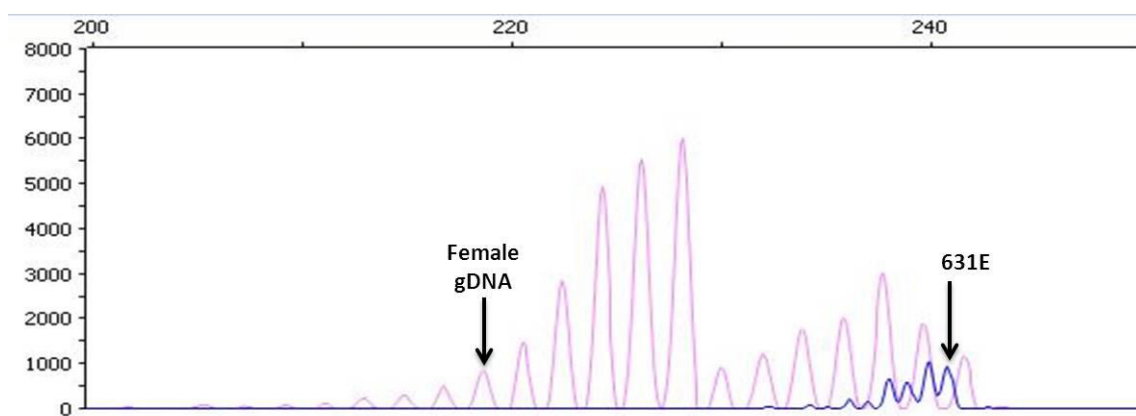
Lane 9= 9151 gDNA

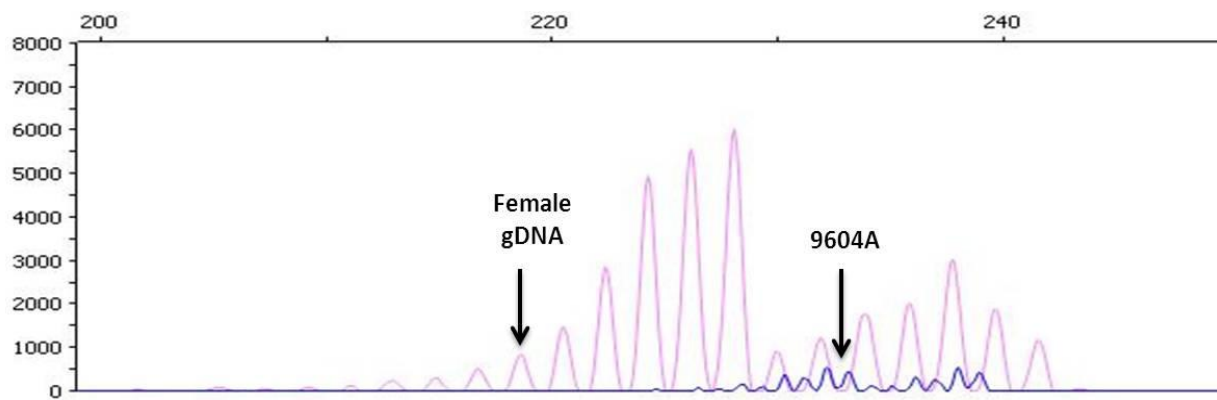
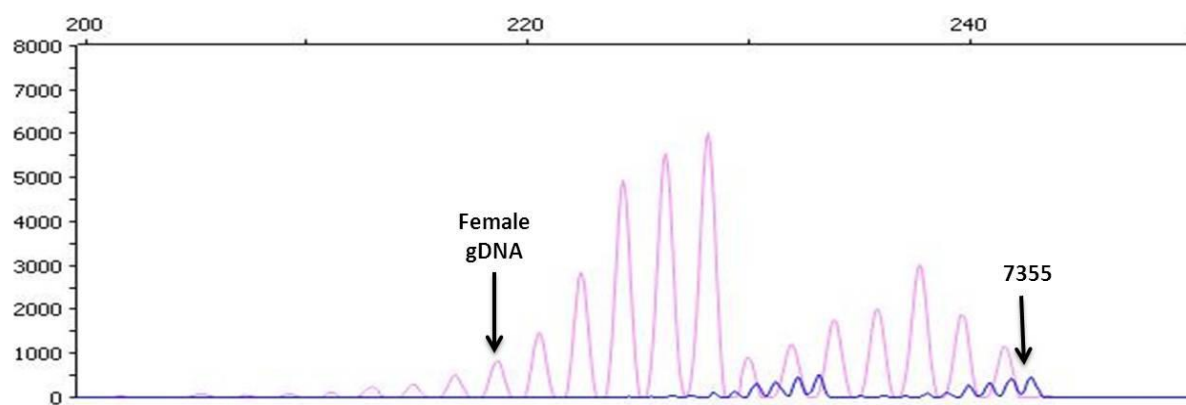
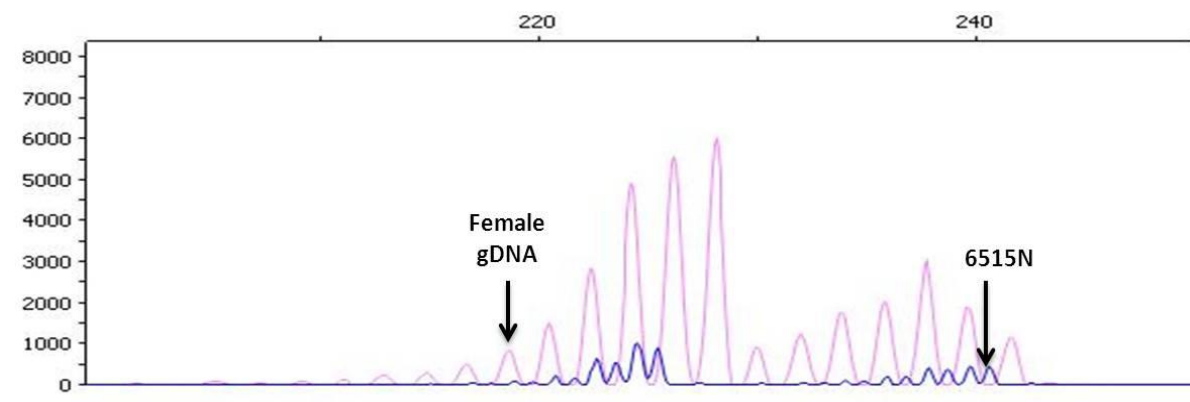
Lane 10= 6534H gDNA

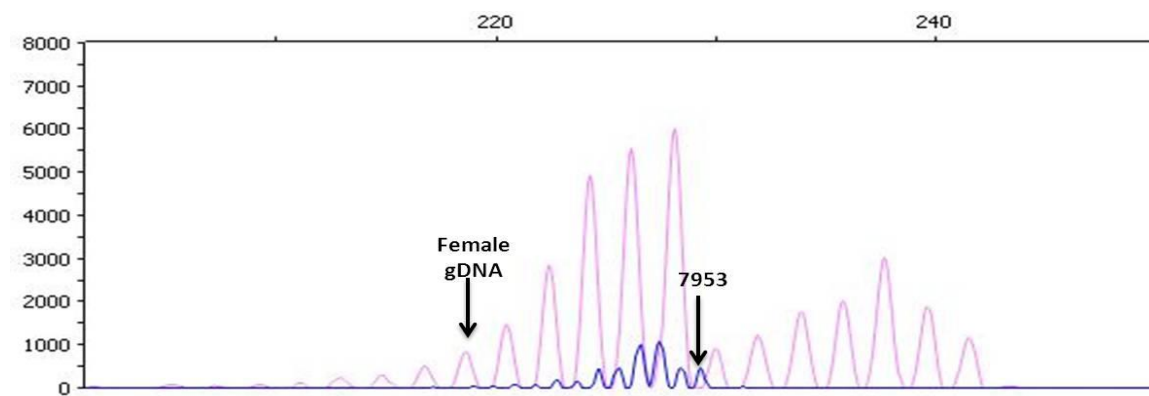
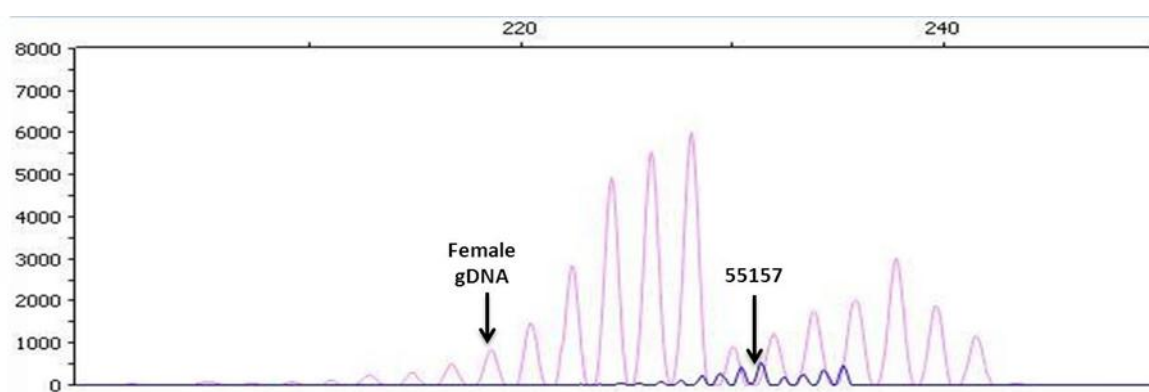
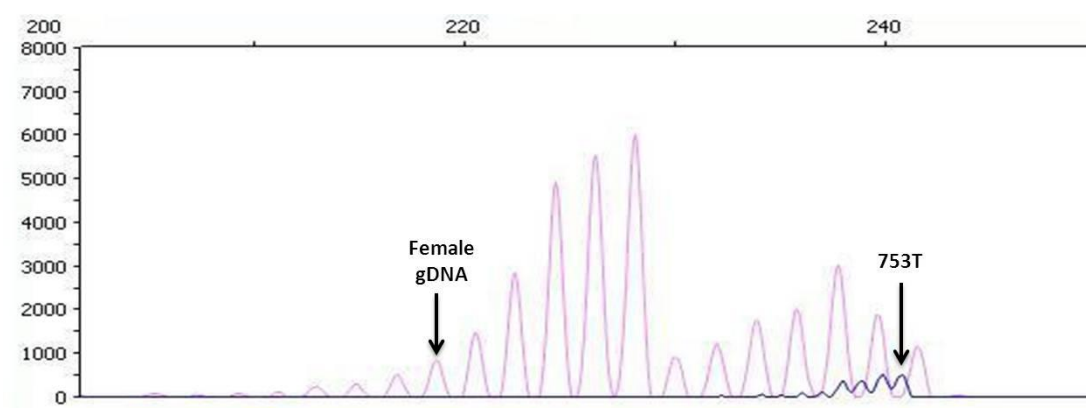
Lane 11= 157Q gDNA

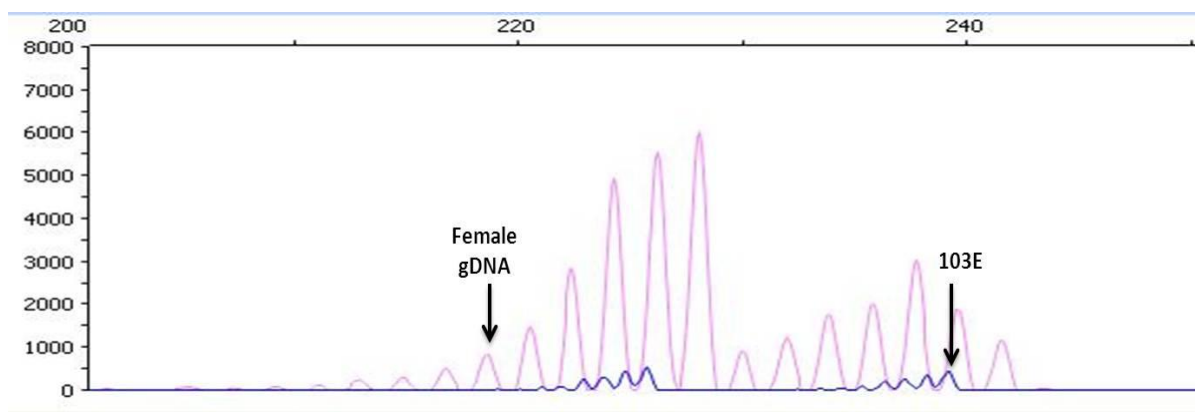
Lane 12= 6517S gDNA

Appendix E: Electropherograms from **1-20** are of 20 different gDNA samples from buffy coat (in blue colour) compared with female gDNA (in pink colour). PCR was performed with D21S1890 FAM labelled external forward and non-labelled external reverse primers at a denaturation temperature of 95°C. This was to provide fragments containing STR for analysis, as in theory this fragment should be 229 bp in size, but as individual may have different numbers of CA repeats the analysis of these fragments might show allelic heterozygosity. Electropherograms (derived from Peak Scanner™ software), Y axis represents signal intensity peak height and X axis represents size in base pairs.

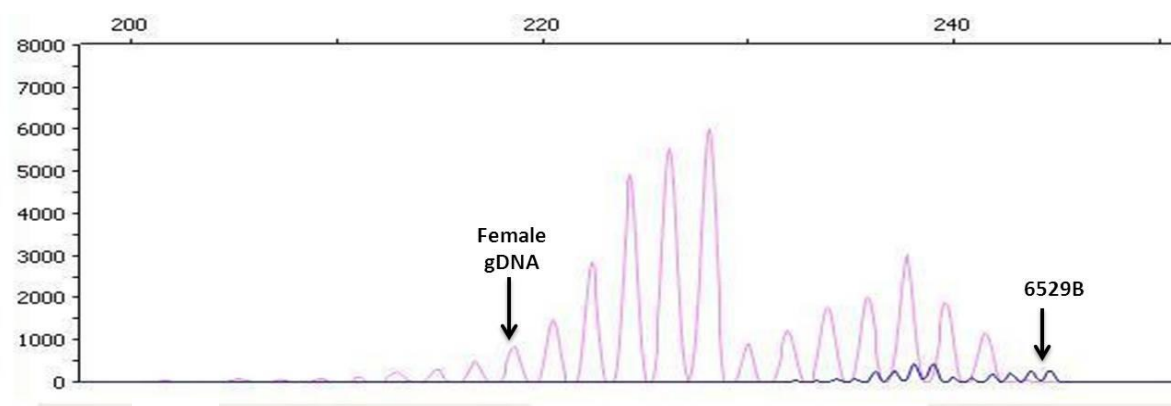




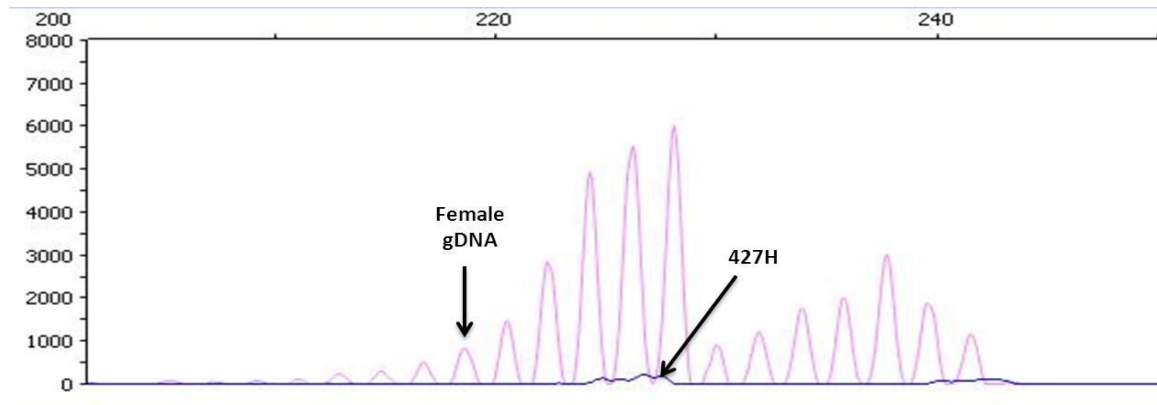




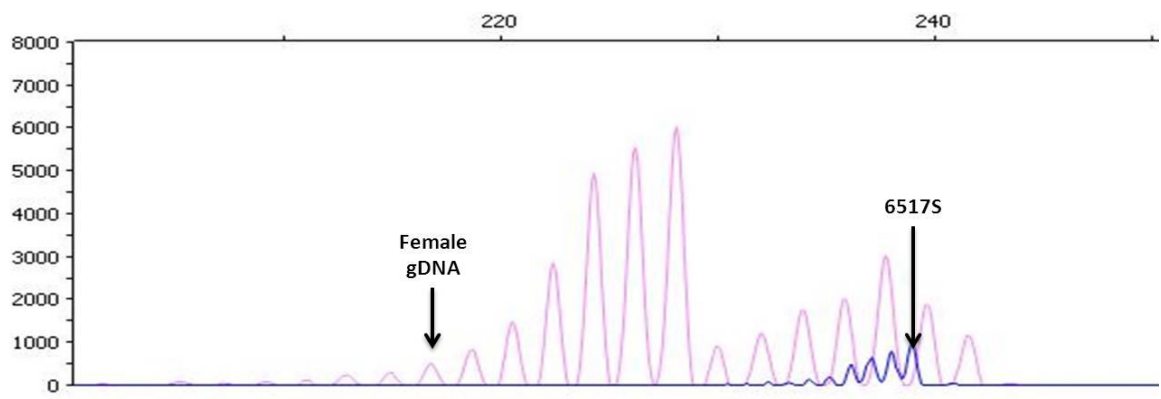
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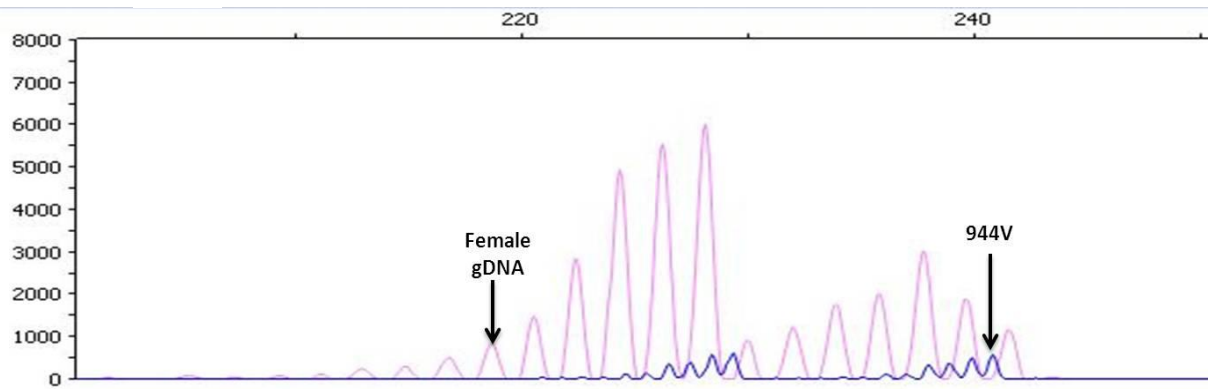
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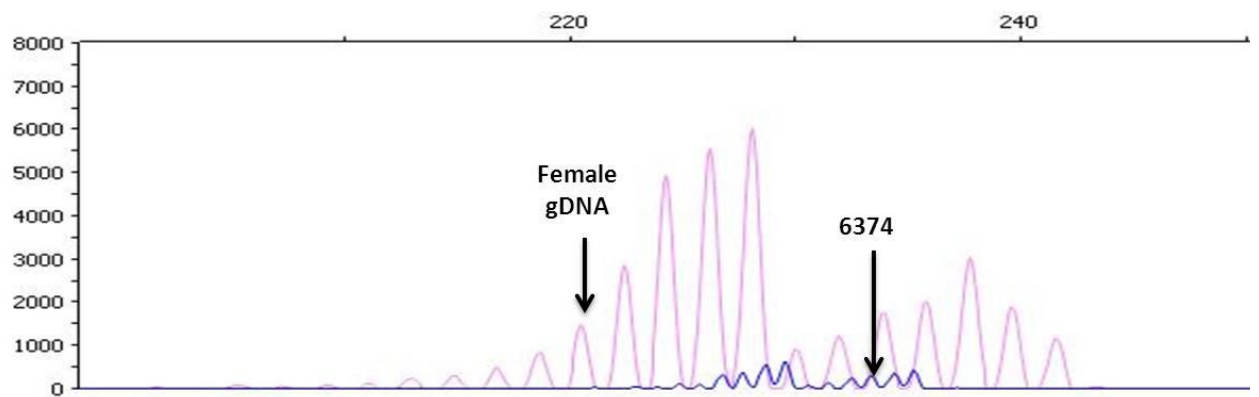
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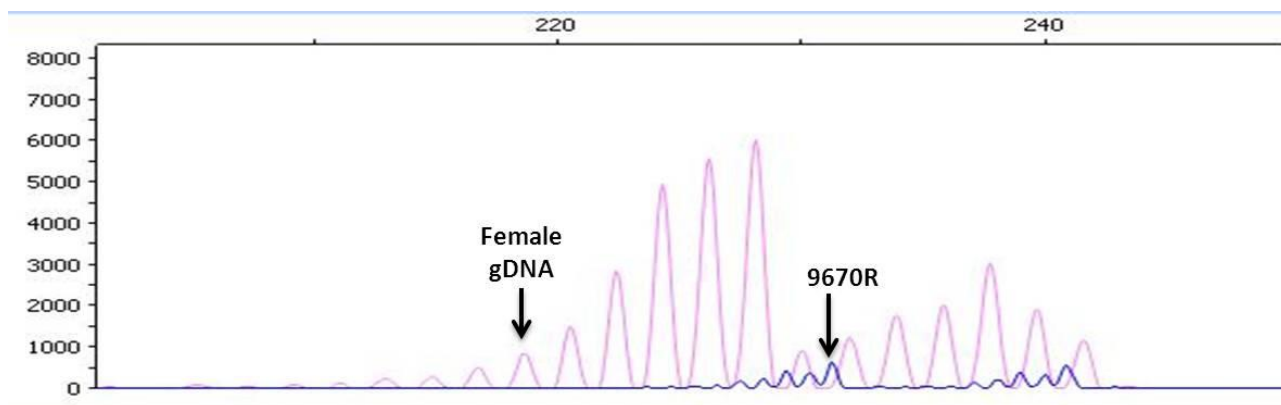
13)



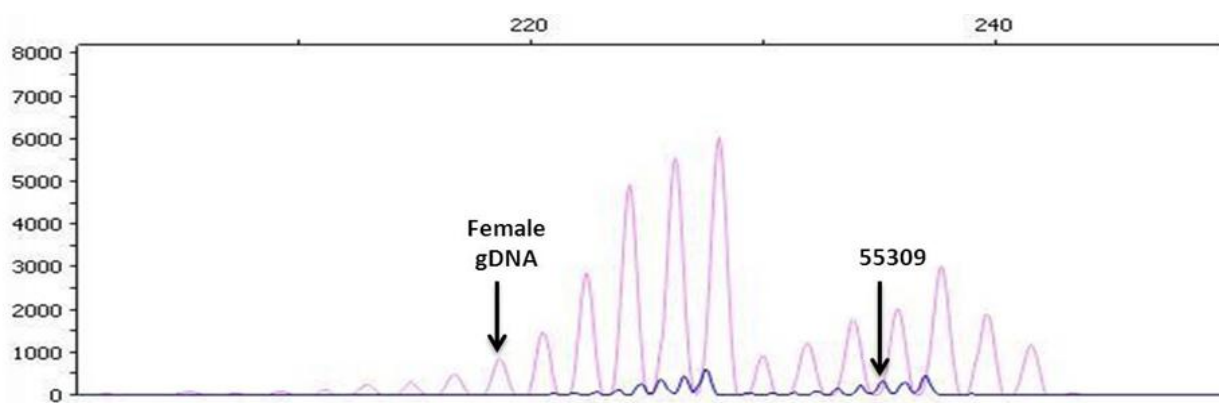
14)



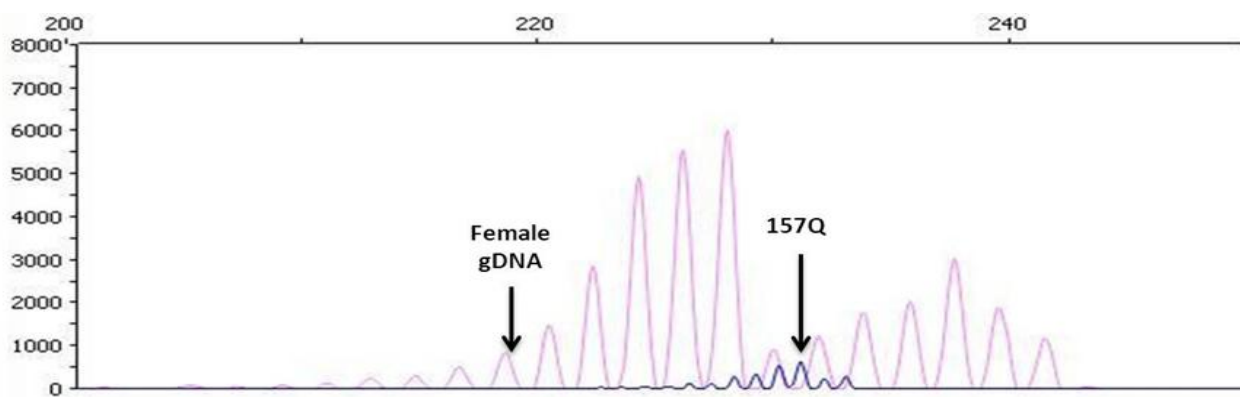
15)



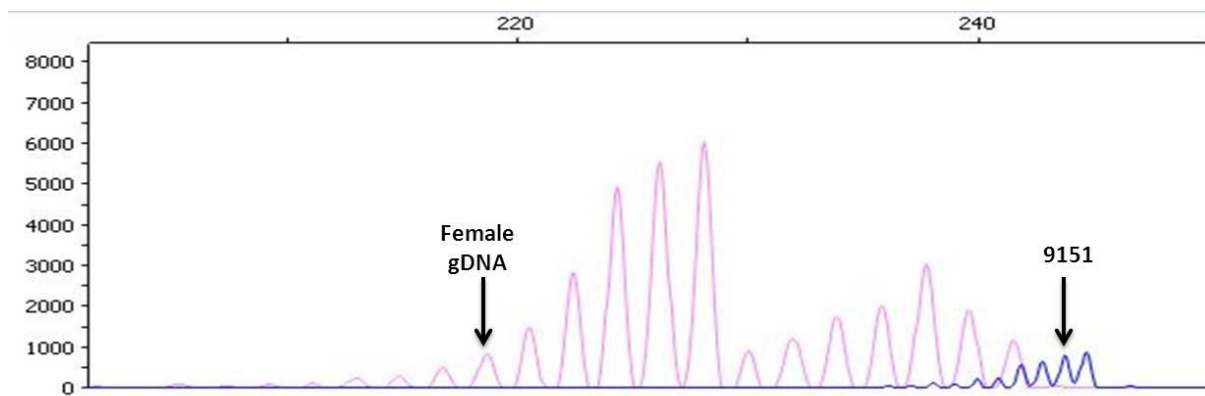
16)



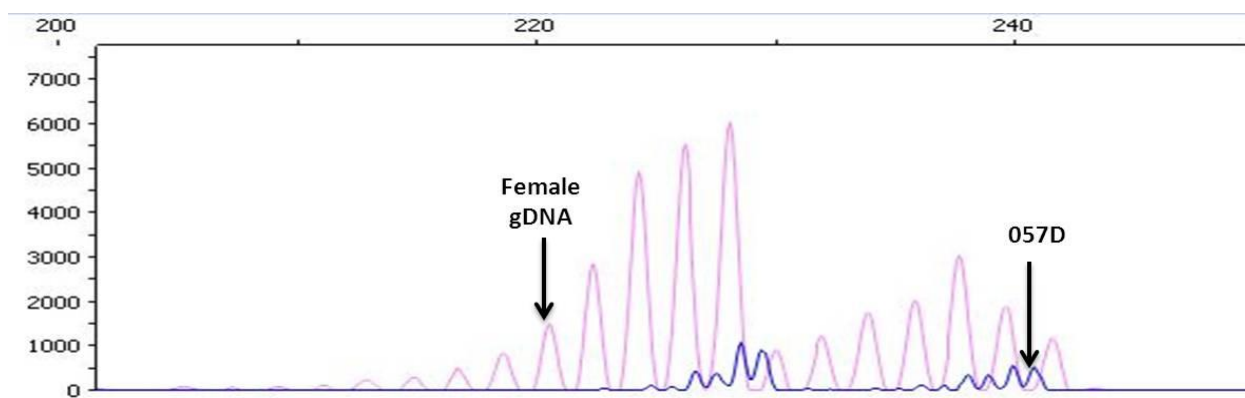
17)



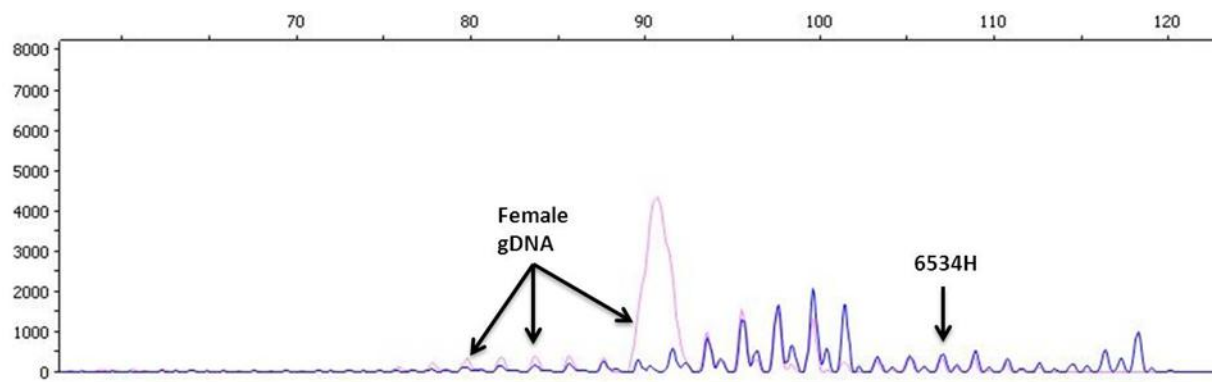
18)



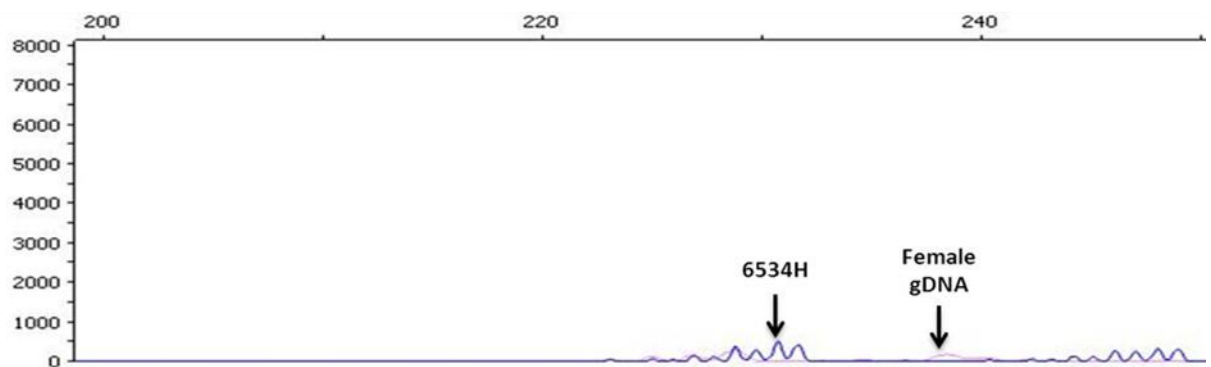
19)



20)

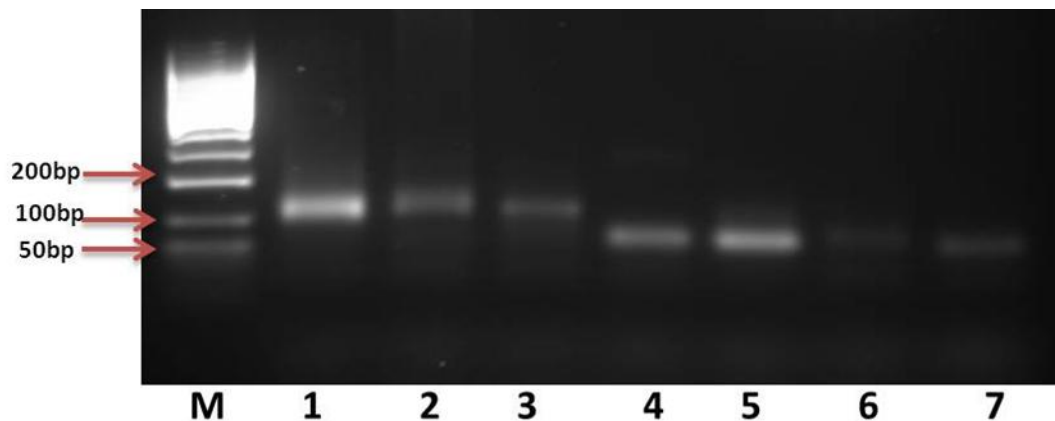


A)

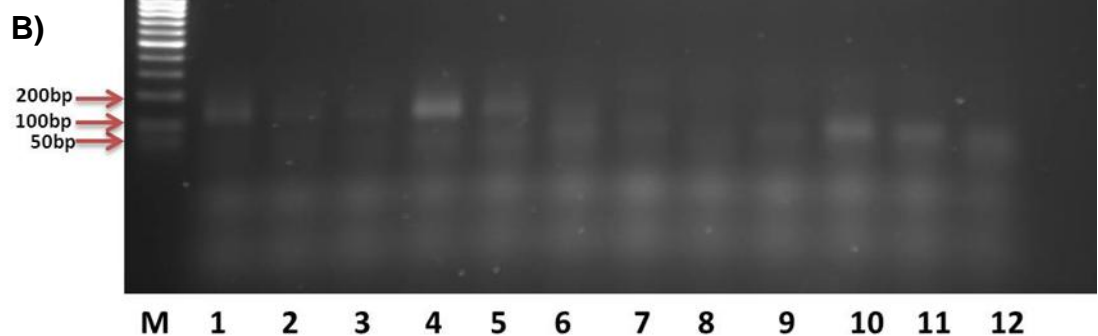
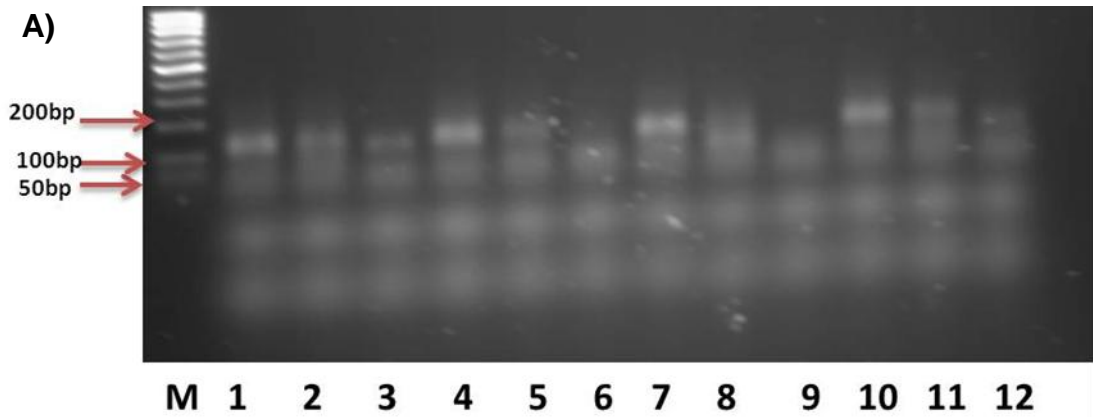


B)

Appendix F: Electropherograms (derived from Peak Scanner™ software), Y axis represents signal intensity peak height and X axis represents in size base pairs. PCR was carried out using AmpliTaq Gold enzyme with fake fetal and fake maternal DNA as templates **A)** with D21S1890 internal forward and reverse primers and **B)** with D21S1890 external forward and reverse primers. PCR with both sets of primers was performed with a denaturation temperature of 95°C.

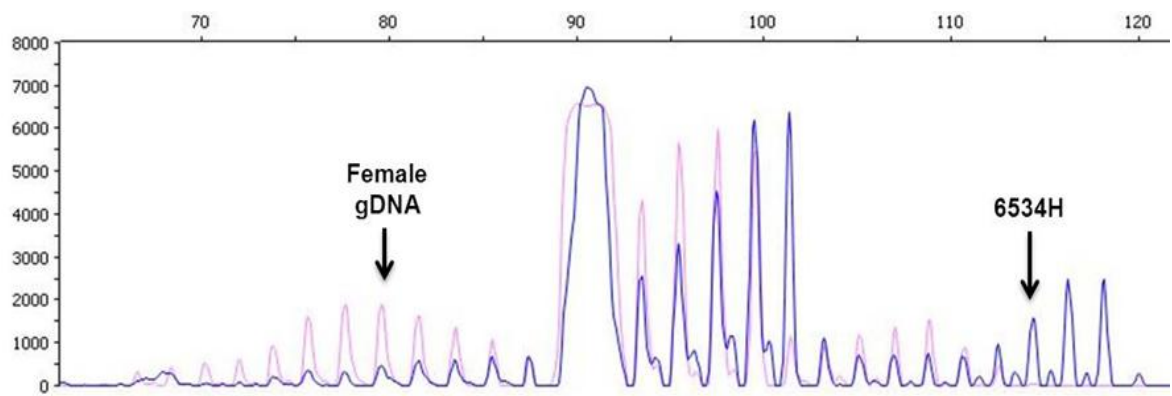


Appendix G: Agarose gel of PCR with FAM labelled D21S1890 internal forward and reverse primers, using AmpliTaq Gold enzyme, Female and male fake fetal DNA and fake maternal DNA were used as templates at denaturation temperatures of 95°C and 80°C. The PCR was carried out with a heating step of master mix at 95 °C for 15 min and after that the templates were added to the master mix. Then the running programme continued as 95 °C/80 °C for 15 sec, 56 °C for 1 min and 4 °C hold. M=DNA ladder, lane 1=female fake fetal DNA at 95°C, lane 2= fake maternal DNA at 95°C and lane 3=NTC. Lane 4=female fake fetal DNA at 80°C, lane 5= male fake fetal DNA at 80°C, lane 6= fake maternal DNA at 80°C and lane 7=NTC (positive contamination)

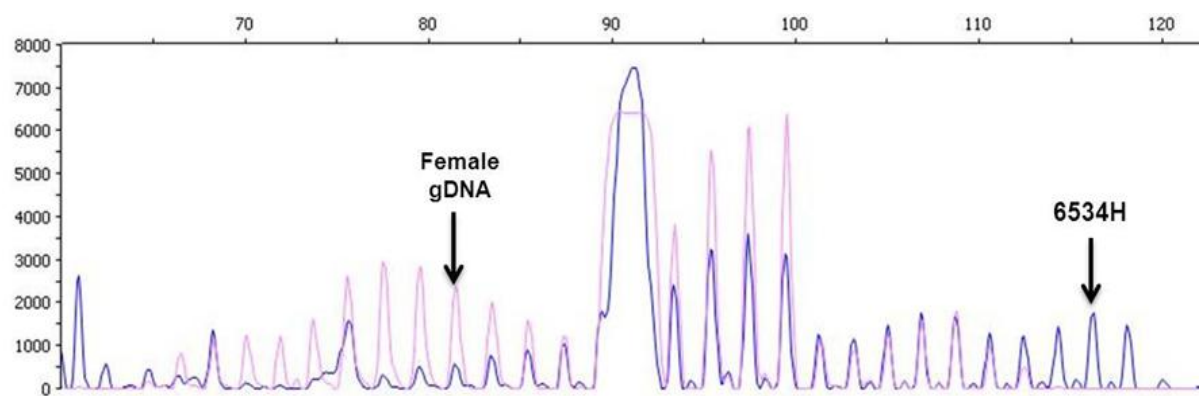


Appendix H: Agarose gel of PCR with Enhancer at denaturation temperatures of 95°C and 80°C. A) TaqMan Fast universal PCR master mix was used with FAM labelled D21S1890 internal forward and reverse primers. B) Same as A but with AmpliTaq Gold. M= DNA ladder

Lane 1= fake fetal DNA	}	TaqMan mastermix at Td 95°C
Lane 2= fake maternal DNA		
Lane 3= NTC		
Lane 4= fake fetal DNA	}	TaqMan mastermix + Enhancer at Td 95°C
Lane 5= fake maternal DNA		
Lane 6= NTC		
Lane 7= fake fetal DNA	}	TaqMan mastermix at critical Td 80°C
Lane 8= fake maternal DNA		
Lane 9= NTC		
Lane 10= fake fetal DNA	}	TaqMan mastermix + Enhancer at critical Td 80°C
Lane 11= fake maternal DNA		
Lane 12= NTC		

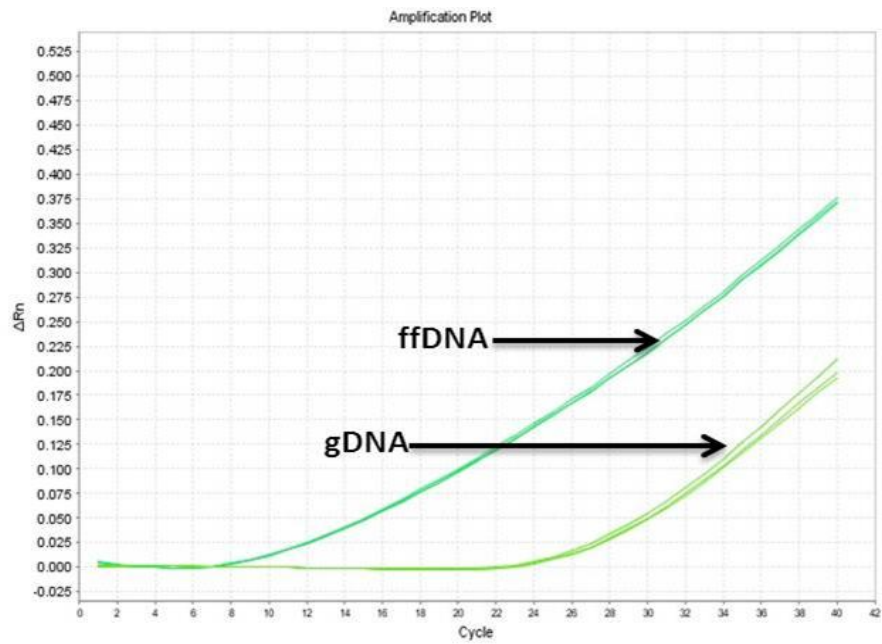


A)

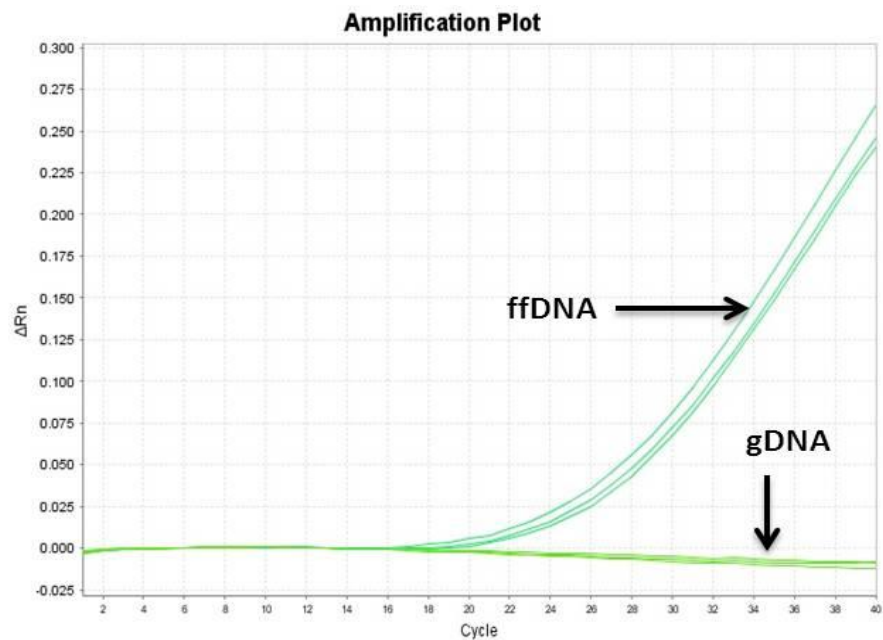


B)

Appendix I: Electropherograms (derived from Peak Scanner™ software), Y axis represents signal intensity peak height and X axis represents size in base pairs. **A)** TaqMan universal master mix with Enhancer, at a denaturation temperature of 95°C. **B)** AmpliTaq Gold enzyme with Enhancer at a denaturation temperature of 95°C.

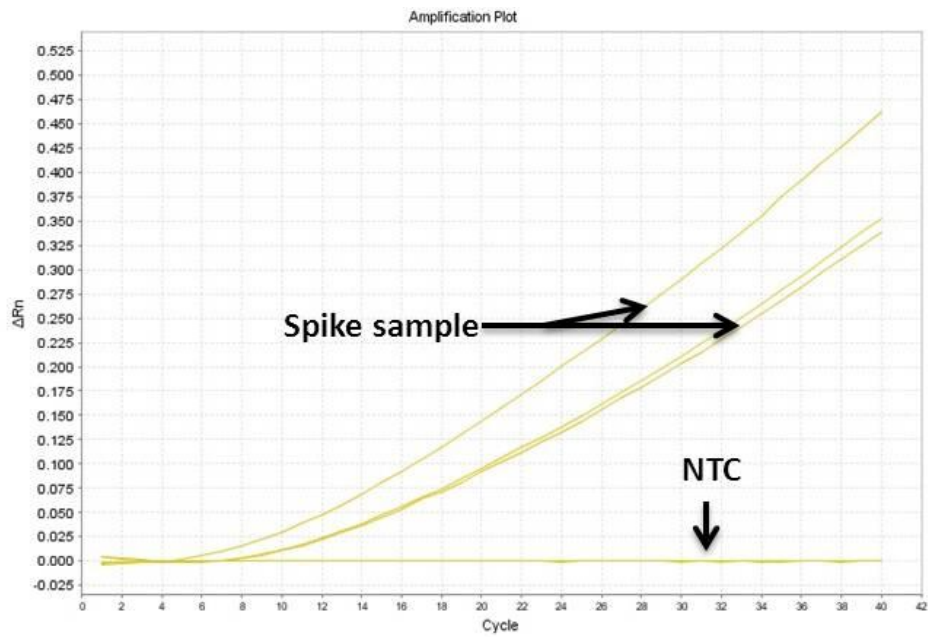


A)

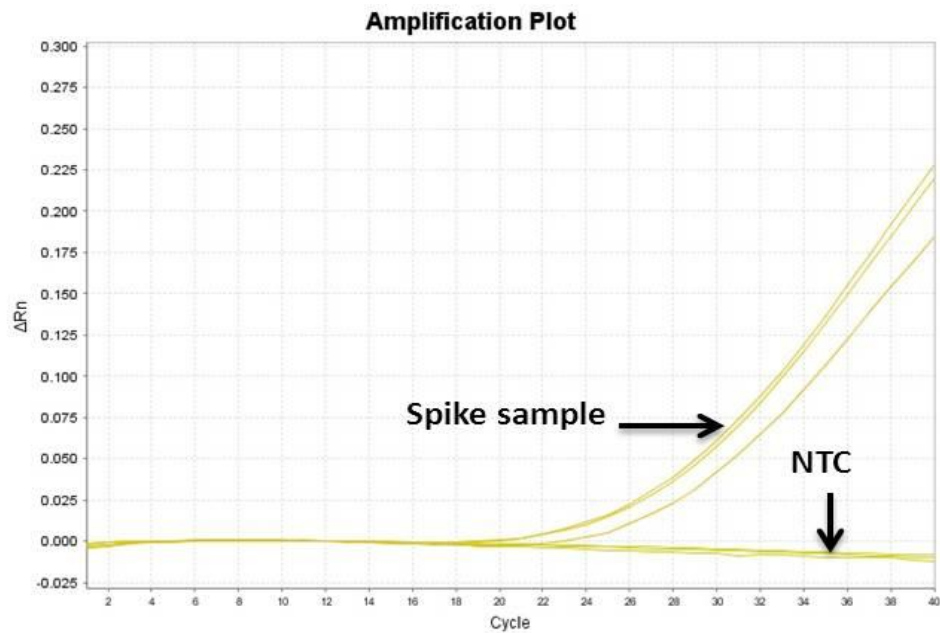


B)

Appendix K1: RT-PCR amplification plot for the spike experiment, showing both templates, fake fetal and fake maternal DNA. **A)** Amplification plot at a denaturation temperature of 95°C, mean Ct value for fake fetal and fake maternal DNA were 10.27 and 25.81 respectively. **B)** Amplification plot at 80°C critical denaturation temperature. Mean Ct value for fake fetal DNA was 25.63 and fake maternal DNA shows no amplification (undetermined mean Ct value).

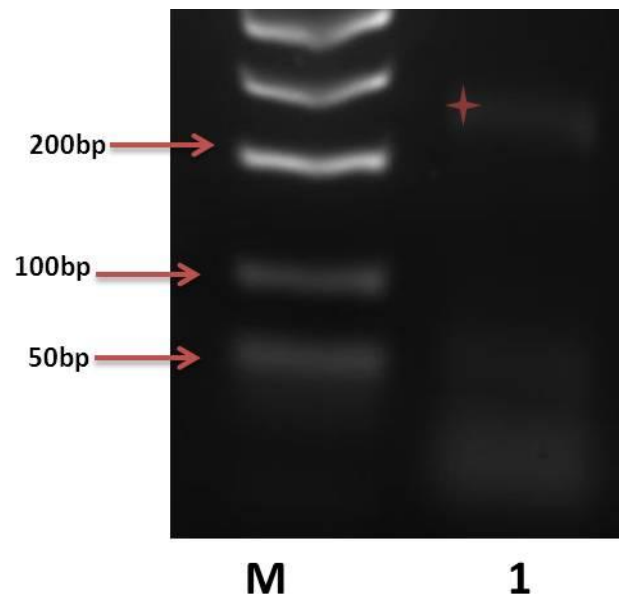


A)



B)

Appendix K2: RT-PCR amplification plot for the spike experiment showing spike sample (95% fake maternal DNA + 5% fake fetal DNA) at denaturation temperatures of 95 °C and 80 °C. **A)** At a denaturation temperature of 95 °C, mean Ct value of spike sample was 9.49. **B)** At critical denaturation temperature of 80 °C, mean Ct value of spike sample was 27.34. At both denaturation temperatures NTC showed no contamination.



Appendix L: Agarose gel of PCR of fake maternal DNA for cloning experiment. D21S1890 external forward and reverse primers were used at a denaturation temperature of 95°C. M= DNA ladder, lane 1= fake maternal DNA (6534H).

[illegible]

Appendix M: Multiple alignment of DNA sequence (Chromosome 21/ Band: 21q22.3, D21S1890) for fake fetal DNA sample at denaturation temperatures of 95°C and 80°C. M13 primers were used for sequencing plasmid DNA samples from two random colonies of fake fetal DNA, one at denaturation temperature of 95°C and other at critical denaturation temperature of 80°C. These samples were compared with reference sequence from D21S1890 with internal forward and reverse primers as start and end points. Yellow sections indicate D21S1890 forward and reverse primers, green sections indicates CA repeat region. These results clearly show no sequence of CA repeats at critical denaturation temperature 80 °C, while at a denaturation temperature of 95 °C shows CA repeat compatible with CA repeat of D21S1890 product.

6.2 Publications

WEBB, A., MADGETT, T. E., MIRAN, T., SILENCE, K., KAUSHIK, N., KIERNAN, M. & AVENT, N. D. 2012. Non-invasive prenatal diagnosis of aneuploidy: Next generation sequencing or Fetal DNA enrichment? *Balkan Journal of Medical Genetics*. (In Press)